PROGESTERONE CONTROL OF HUMAN ENDOMETRIAL CELLS

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Abstract

The coordination of events and cellular interactions within the uterus is vital to the establishment of pregnancy, a process constrained to a narrow window of time within the ovarian cycle. The transformation of the endometrium into decidua is one such event and is considered essential to embryo implantation and to the maintenance of pregnancy. In humans these changes are most dramatic in the stromal compartment and are influenced by progesterone, secreted by the Corpus Luteum (CL). This hormone is believed to be the main factor inducing such differentiation in the oestradiol-primed stroma. However, mediators with the ability to activate the protein kinase A/cAMP pathway, such as Prostaglandin E_2 (PGE_2) and relaxin, are potent inducers of decidualisation when administered alongside progesterone in endometrial stromal cells (ESCs) *in vitro*. Uterine Natural Killer (uNK) cells increase in number in secretory phase endometrial stroma, implying the control of progesterone on their expansion. However, they lack the nuclear progesterone receptor and growth and differentiation may depend on interactions with ESCs. uNK cells replicate upon Interleukin-15 (IL-15) administration *in vitro* and this cytokine is expressed in the epithelial and stromal cells. The aims of this research project have been to investigate *in vitro* decidualisation of ESCs and regulation of IL-15 and uNK cells in order to extrapolate how ESCs and uNK cells may interact during the secretory phase and early pregnancy.

The present study has explored the factors involved in decidualisation using primary human ESC cultures. Quantitative real-time PCR (Q RT-PCR) and Enzyme-linked Immunoabsorbant Assays (ELISA) have been used to investigate effective *in vitro* stimuli of decidualisation. A combination of treatment with a progestin and either 8-Bromo cAMP or PGE_2 was capable of stimulating decidualisation in ESC cultures as determined by increases in two markers of this process, prolactin and insulin-like growth factor-binding protein-1 (IGFBP-1). Further analysis has revealed the changes taking place within the PGE_2 pathway in decidualising ESCs, including an upregulation in the

EP_2 prostaglandin receptor messenger RNA (mRNA) upon treatment with 8-Bromo cAMP plus a progestin.

The results present here have demonstrated a rise in IL-15 mRNA levels in parallel with *in vitro* decidualisation. It appears that both progesterone and the intracellular messenger, cAMP, are involved in decidualisation and IL-15 expression. IL-15 secretion from the cells is shown to be IFN-γ dependent. The expression of IL-15 and interferon-γ (IFN-γ) mRNA across the menstrual cycle has been established. Immunohistochemistry was used to determine IL-15 expression during simulated early pregnancy compared with normal luteal controls and has shown that secretions of the CL, including progesterone and/or relaxin, have the ability to increase IL-15 expression *in vivo*. Primary cultures of human uNK and peripheral blood NK cells have been used for studying the T helper 2-type cytokine IL-10 which is believed important for the support of early pregnancy. In response to PGE_2 treatment, uNK cells expressed and secreted raised levels of IL-10, an anti-inflammatory cytokine.

Further investigation into the interactions between the convergence of the cAMP and progesterone intracellular pathways and their receptors would be important in clarifying the exact mechanisms controlling ESC decidualisation and IL-15 regulation. The interactions between ESCs and uNK cells need to be clarified further to assess the roles of uNK cells in reproductive processes.

Contents

Declaration

Except where due acknowledgement is made by reference the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

Carolyn Dunn

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Abbreviations

AA	arachidonic acid
ABC	avidin biotin peroxidase detection system
ANOVA	analysis of variance
ART	assisted reproductive technology
AS	anti-sera
ATP	adenosine triphosphate
α-2 M	α-2 macroglobulin
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
cAMP	adenosine-3',5'-cyclic monophosphate
CAT assay	chloramphenicol acetyl transferase
CD	cluster determinant
CD40L	CD40 ligand
cDNA	complimentary DNA
c-fms	receptor of M-CSF
CL	corpus luteum
COX-1/2	cyclo-oxygenase-1/2
CSF-1	colony stimulating factor-1
CSM	cell separation medium
Ct	threshold cycle
DAB	3,3'-diaminobenzidine
DEPC	Diethyl pyrocarbonate
dH_2O	distilled water
DSC	decidualised stromal cell
ECM	extracellular matrix
EDTA	ethylenediaminetetracetic acid
EGF	epidermal growth factor
ELISA	enzyme linked immunoabsorbant assay

EP_{1-4}	PGE_2 receptors 1-4
ER-α/β	oestrogen receptor-α/β
ESC	endometrial stromal cell
FACS	fluorescent activated cell sorter
FAM	6-carboxy-fluorescein
FCS	fetal calf serum
FSH	follicle stimulating hormone
FITC	fluorescein isothiocyanate conjugate
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte/macrophage-colony stimulating factor
GPCR	G-protein coupled receptor
HBD 1-4	human beta defensins 1-4
hCG	human chorionic gonadotrophin
HLA-DR	human leukocyte antigen
ICAM	inflammatory cell adhesion molecule
IFN-α/β/γ/τ	interferon-α/β/γ/τ
IGF	insulin-like growth factor
IGFBP-1/2 etc	insulin-like growth factor binding protein-1/2 etc
IgG	immunoglobulin
IL-1/2 etc	interleukin-1/2 etc
IL-1R/11R etc	IL-1/11 receptor etc
iNOS	inducible nitric oxide synthase
IRF-1	interferon regulatory factor-1
IUGR	intra uterine growth restriction
KDR	Type 2 receptor of VEGF
LGL	large granular lymphocyte
LH	luteinising hormone
LIF	leukaemia inhibitory factor
LMP	last menstrual period
LNG-IUS	levonorgestrel-releasing intra uterine system

LPD	luteal phase defect
LSP	long signal peptide
MCP-1	monocyte chemoattractant protein
M-CSF	macrophage-colony stimulating factor
MHC	major histocompatibility complex
MIP-1α	macrophage inflammatory protein-1α
MoxB	methyl oximating B solution
MP	multi-purpose
MPA	medroxyprogesterone acetate
MMP	matrix metalloproteinase
MRP-4	multi-drug resistant protein-4
NBF	neutral buffered formalin
NFκB	nuclear factor κ B
NHS	normal horse serum
NK	natural killer (cell)
NSAIDs	non-steroidal anti-inflammatory drugs
NSB	non-specific binding
PB	peripheral blood
PBMC	peripheral blood monocyte cells
PBS	phosphate buffered saline
PDE3a/4b	phophodiesterase3a/4b
PG	prostaglandin
PGD_2	prostaglandin D_2
PGDH	prostaglandin dehydrogenase
PGH_2	prostaglandin H_2
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostaglandin I_2
PGTP	prostaglandin transport protein
PKA	protein kinase A

PLA_2	phospholipase A_2
PLSD	protected least squares difference
PR-A/B/C	progesterone receptor-A/B/C
PMA	phorbol 12-myristate 13-acetate
Prl	prolactin
Prl-R	prolactin receptor
Q RT-PCR	quantitative reverse transcription-polymerase chain reaction
RCLB	red blood cell lysis buffer
RIA	radioimmunoassay
rpm	revolutions per minute
RSB	RNA storage buffer
RU486	mifepristone
SLPI	secretory leukocyte protease inhibitor
SPC	streptavidin peroxidase conjugate
SSP	short signal peptide
TBS	tris buffered saline
TGF-β	transforming growth factor-β
Th-1/2	T helper-1/2
TIMP	tissue inhibitor of MMP
TNF-α	tumour necrosis factor-α
TXA_2	thromboxane A_2
uNK	uterine natural killer (cell)
VEGF	vascular endothelial growth factor

1. Literature Review

1.1 The Uterus and the Menstrual Cycle

The uterus has the role of supporting and retaining the fetus throughout pregnancy. The organ is lined by a glandular tissue layer termed the endometrium and in the non-pregnant state the functional layer of the human endometrium undergoes cyclical regeneration and degeneration. This takes place over a 28 day period and is primarily determined by the ovarian hormones, oestrogen and progesterone. A progressive and sequential maturation of the endometrium is essential preparation for implantation of the blastocyst. There are three major phases, namely menstruation, proliferation and secretion (figure 1.1). The secretory phase is surprisingly highly regular in length, even if there is individual variation in proliferative phase length. The functional layer of the endometrium is shed via the process of menstruation if implantation fails to occur. This layer is then replenished from the basal endometrial layer and the process begins once more. The major cyclical changes in response to progesterone and oestrogen have been described by Noyes *et al* 1950 (Noyes *et al.*, 1950) (figure 1.2).

1.1.1 The Proliferative Phase

The proliferative phase lasts from day 4 to day 14 of the cycle and is oestrogen dominated. Oestrogen receptors are present in the glandular and stromal compartments as are progesterone receptors during the follicular phase (Lessey *et al.*, 1988; Snijders *et al.*, 1992; Wang *et al.*, 1998). The primary source of oestrogen is the developing Graaffian follicle. Follicle stimulating hormone (FSH) released from the pituitary gland stimulates maturation of one or more of these follicles. The oestrogen released stimulates proliferation of the endometrium and this is reflected by a variety of changes. New glands form and are straight and tubular in structure and along with the stroma, regenerate from the residual basal endometrium. The nuclei of the glands become prominent and increase in mitotic activity. In the compact stromal compartment mitoses are also abundant. The stroma becomes highly vascularised and the arteries become

spiral in formation. As the luteinising hormone (LH) surge on day 14 approaches the glands become pseudo-stratified and tall and columnar in shape.

1.1.2 The Secretory Phase

The secretory phase commences from day 16 to 25 and is progesterone dominated. The continued release of LH from the pituitary induces the luteinisation of the theca and granulosa cells of the corpus luteum (CL). The granulosa lutein cells secrete progesterone, maintaining the secretory transformation of the endometrium.

In the early secretory phase the glands develop subnuclear vacuoles of glycogen and they become convoluted. The gland mitoses decrease at this point and are absent after day 18. The glandular glycogen deposits begin to appear on the apical side of the cells around day 18 of the cycle and continues to increase with maximal secretions being released around days 20 and 21 before exhausting their stores. The tortuosity of the glands increases in the mid to late secretory phase. Stromal oedema increases across the secretory phase, peaking in the mid-section. During the mid to late secretory phase the stromal cells begin to change both structurally and functionally. This predecidual change is initially observed around the spiral arterioles before it spreads to other regions of the stroma. The stromal cells undergo a decidual reaction in preparation for implantation and progressively forms the decidua during pregnancy. Pinopodes are expressed on days 19-21 on the apical surface of the luminal uterine epithelium. The timing of their appearance is progesterone-dependant and they appear around the time of implantation (Martel *et al.*, 1991; Nikas *et al.*, 1995; Nikas *et al.*, 1997).

In the late secretory phase, an infiltration of leukocytes invades the endometrium. Although macrophages and lymphocytes accumulate, the major leukocyte cell type in the secretory phase is the uterine Natural Killer (uNK) cell. These cells remain abundant in the first trimester of pregnancy and will be discussed in more detail in section 1.2.2.

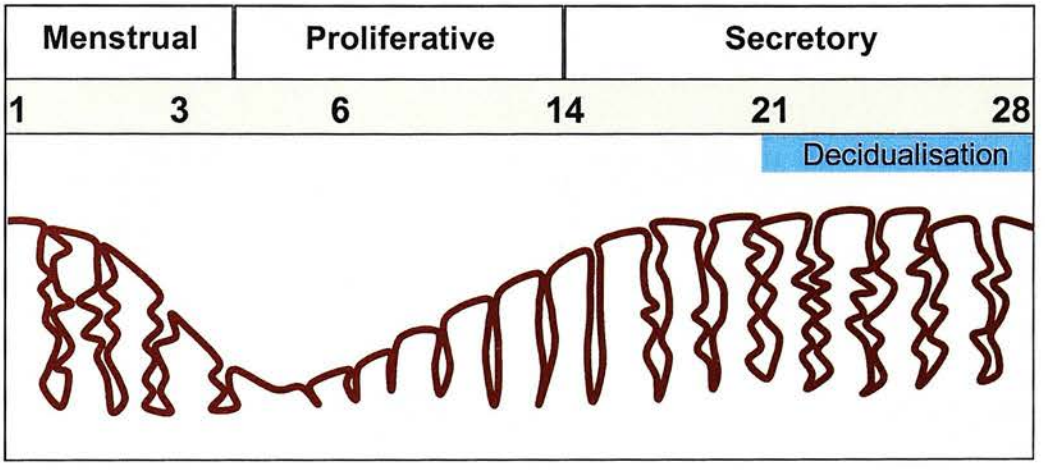
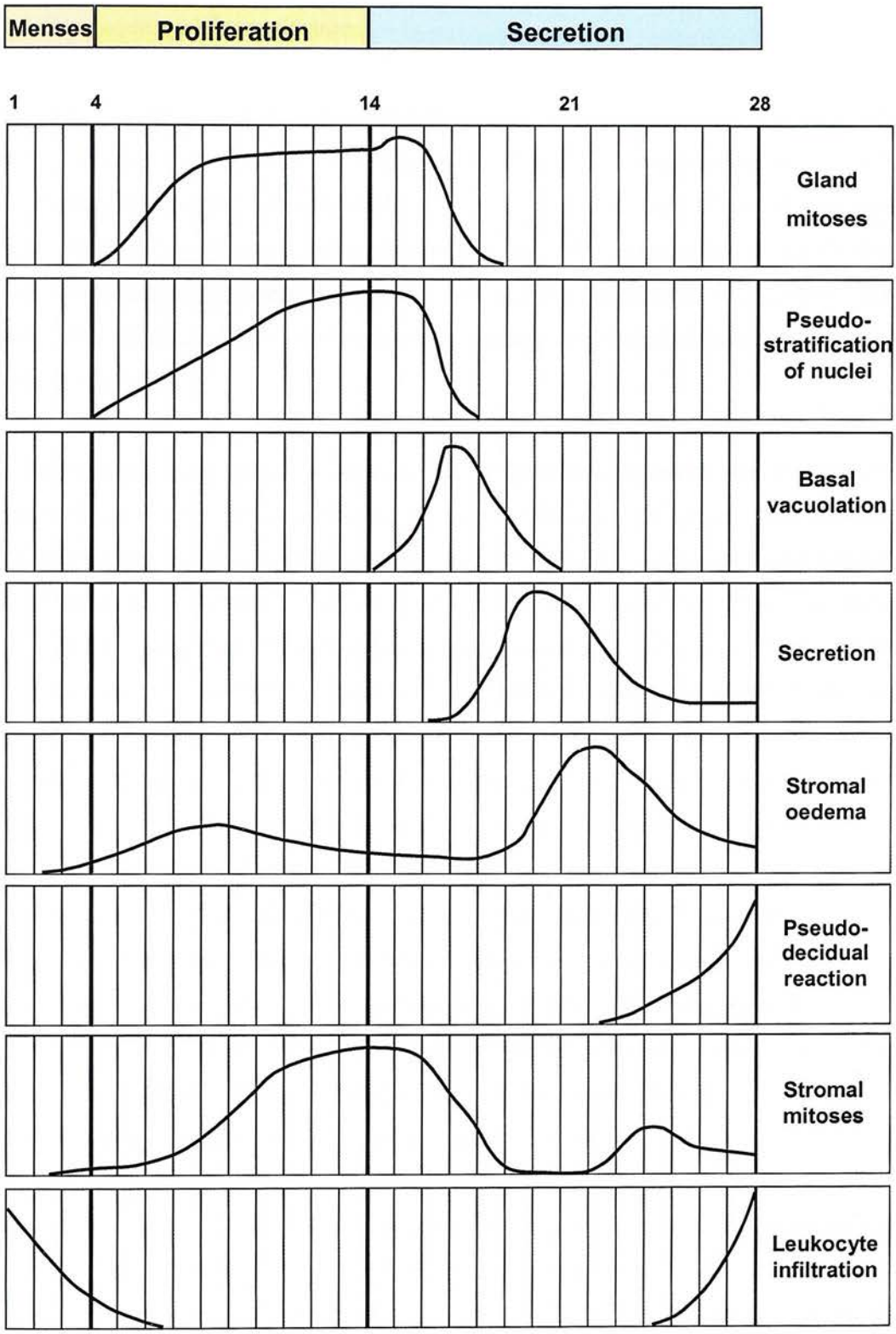


Figure 1.1

Diagrammatic representation of the cyclical nature of degeneration and regeneration of the functional endometrial layer with the major stages being highlighted. This is based on the "classic" 28 day cycle in humans.

Figure 1.2

The changes that take place in the human endometrium across the menstrual cycle based on the "classic" 28 day model as described by Noyes *et al* 1950 (Noyes *et al.*, 1950).


Menses
Proliferation
Secretion
1
4
14
21
28
Gland mitoses
Pseudo-stratification of nuclei
Basal vacuolation
Secretion
Stromal oedema
Pseudo-decidual reaction
Stromal mitoses
Leukocyte infiltration

1.1.3 Oestrogen and Progesterone

The co-ordinated actions of the steroid hormones, oestrogen and progesterone, are pivotal to succinct female reproductive functions. They act throughout the reproductive tract, including the hypothalamus, pituitary, uterus and ovary. The effects of oestrogen are not restricted to the reproductive system proving to be crucial in bone remodelling (Prior, 1990) and is involved in peripheral vasoreactivity (Hashimoto *et al.*, 1995). The effects of hormones and hormone replacement therapy on vascular function is discussed by Ganz (Ganz, 2002).

Oestrogens act on the uterus to increase cellular proliferation across the proliferative phase and also act to prime progesterone receptors (PR) during this phase. The presence of a functioning PR is crucial to fertility in mice with PR knockouts being infertile displaying defective ovulation, implantation and stromal cell decidualisation (Conneely *et al.*, 2002). The actions of oestrogen and progesterone are mediated via their cognate receptors located intracellularly in the nuclear compartment primarily, and in turn, the ligand may act to control levels of receptor expression. Thus, the hormones themselves are capable of modifying the concentration of receptors at the end-organ and this will partly determine the extent of their actions. Hormone-bound receptors interact to modulate gene transcription resulting in a function change. There are two types of oestrogen receptor (ER), ERα (Green *et al.*, 1986) and ERβ (Kuiper *et al.*, 1996; Mosselman *et al.*, 1996), and two isoforms of the progesterone receptor (PR) exist, PR-A and PR-B (Horwitz *et al.*, 1983; Lessey *et al.*, 1983). PR-A and PR-B arise from different promoters of the same gene located on chromosome 11 (Clarke *et al.*, 1990; Kastner *et al.*, 1990). PR-B is identical to PR-A with the exception that it differs in length by being an additional 164 amino acids in length than PR-A (Horwitz *et al.*, 1983). Upon binding its receptor, progesterone triggers a conformational change and receptor dimerisation. Heat shock proteins also become dissociated from the receptor in the binding procedure. A third PR has been identified and termed PR-C and may be able to alter the transcriptional activity of PR-A and PR-B (Wei *et al.*, 1990; Wei *et al.*,

1996). In addition, more recently a fourth novel, truncated PR has been cloned from human adipose and aortic cDNA libraries and is believed to have a non-genomic action (Saner *et al.*, 2003). The functional importance of this receptor with context to the endometrium is currently under investigation.

1.1.3.1 Receptor Cross-talk

Communication also takes place between the oestrogen and progesterone receptors. PRs are under the dual control of both oestrogen and progesterone. Experiments involving rat uterine cells demonstrated that when either a progestin or anti-progestin is bound to either PR-A or -B a resulting inhibition in oestradiol's ability to stimulate ER activity is reported (Katzenellenbogen, 2000). Exposure of the endometrium to high dose progestins, as with the Levonorgestrel-releasing intrauterine system (LNG-IUS), down-regulates the ER and PR-A and PR-B in both the glands and stroma (Critchley *et al.*, 1998b) implying a dependency of ER levels on progesterone and its receptors.

1.1.3.2 Immunohistochemical Localisation

Immunohistological studies within the human endometrium have described the cyclical variation of oestrogen (ER) and progesterone receptor (PR) levels in both functional and basal regions and within the epithelial and stromal compartments (Garcia *et al.*, 1988; Lessey *et al.*, 1988; Critchley *et al.*, 1993; Snijders *et al.*, 1996; Wang *et al.*, 1998) (Summarised in table 1.1). They have demonstrated the nuclear locality of both steroid receptors. PR-A and PR-B are located in the glands and stroma during the proliferative phase but only the PR-A isoform is present in the functional layer across the secretory phase and early pregnancy and is localised to the stromal cells (Wang *et al.*, 1998). This implies that PR-A is responsible for the luteal phase actions of progesterone within the stroma and in particular on decidualisation. Immunoexpression of ERα increases in the functional layer across the proliferative phase and reaches a peak in the late proliferative

endometrium. The ER in the glands is down-regulated in the secretory phase by the rising progesterone levels. This reduction of ER can be blocked by administration of the anti-progestin, RU486, when applied early in the secretory phase confirming the role of progesterone (Maentausta *et al.*, 1993; Cameron *et al.*, 1996). A decline in ERα occurs in glandular and stromal compartments of the functional layer across the secretory phase. *In situ* hybridisation has located the two ER isoforms within the human endometrium (Matsuzaki *et al.*, 1999). The same expression pattern for ERα was observed as with the immunohistological studies. ERβ mRNA was detected in both glands and stroma with decreased expression in the glandular region in the late secretory phase functional layer (Critchley *et al.*, 2001a; Lecce *et al.*, 2001). The ERβ was also localised to endothelial cells implying an influence on vascular regulation. In women with compromised fertility and receiving clomiphene citrate "anti-oestrogen" therapy, the low pregnancy rates achieved were correlated with low oestrogen receptor concentrations in the preovulatory endometrium (Ohno *et al.*, 1998).

A study *in vitro* has shown that during decidualisation the PR-A is more abundant than PR-B. However, they found a reduction in the PR-A with progression of the decidualisation process and this reduction was accelerated by addition of a synthetic progestin (Brosens *et al.*, 1999). In addition, transient transfection of either PR-A or PR-B caused an inhibition of decidual prolactin (Prl) promoter-reporter construct in response to cAMP. Following insertion of LNG-IUS both isoforms of the PR are downregulated in the glands and the stroma (Critchley *et al.*, 1998b) implying regulation of both isoforms by their ligand. It appears to be PR-A that is essential to uterine and ovarian reproductive processes, at least in the murine uterus (Conneely *et al.*, 2002). They discovered that in the chick oviduct PR-A and PR-B are produced by translation at two distinct AUG signals encoded by a single gene and could therefore selectively ablate either of these isoforms (Conneely *et al.*, 1987). Mouse knock-outs for PR-A exhibited infertility, whilst the necessity of the PR-B isoform is restricted to the mammary gland (Conneely *et al.*, 2002).

Table 1.1

Relative immunostaining intensity for oestrogen and progesterone receptors in the functional and basal endometrium across the proliferative and secretory phases of the cycle. Negative immunostaining is represented by (-), positive immunostaining is represented by (+) or (++) depending on intensity. Adapted from Critchley 2000 (Critchley, 2000).

	Proliferative phase		Secretory phase	
	Glands	**Stroma**	**Glands**	**Stroma**
Functional				
PR-A+B	++	++	-	++
PR-B	++	++	-	-
ERα	++	++	-	-
ERβ	++	++	++(+)	++
Basal				
PR-A+B	++	++	+	++
ERα	++	++	+	+
ERβ	++	++	++	++

1.1.4 Regulation of Menstruation

If implantation fails to occur, the functional layer of the endometrium is shed by the process of menstruation in response to declining progesterone levels as a result of CL demise. The work of Markee 1940 on the rhesus monkey provides much of our present understanding of the vascular changes occurring during menstruation with the determination that progesterone and oestrogen are major controlling factors over these structural changes. It was known that progesterone acts following oestrogen priming and then is withdrawn in the lead up to menstruation. The effects of these hormonal changes were studied in endometrial fragments transplanted into the eye of the rhesus monkey, where changes in the vasculature could be observed (Markee, 1940). Vessels become coiled across the luteal phase to form the spiral arterioles and upon withdrawal of progesterone these become vasodilated followed by vasoconstriction (Markee, 1940). This results in lesions and breakdown of the tissue. The vasoactive products found in menstrual fluid have been identified as being PGE_2 and $PGF_{2\alpha}$ (Lumsden *et al.*, 1983). Hormones have been implicated in regulating production of these prostaglandins (PG) in the human endometrium via explant studies (Abel *et al.*, 1980; Abel *et al.*, 1983) and a more detailed account of their regulation is discussed in section 1.4.1. $PGF_{2\alpha}$ is a vasoconstrictive agent and has been implicated in the initiation of menstruation (Baird *et al.*, 1996). During the mid luteal phase prostaglandin dehydrogenase activity (PGDH) is high relative to the proliferative phase and therefore mediates metabolism of PGE_2 and $PGF_{2\alpha}$ (Casey *et al.*, 1980; Critchley *et al.*, 1998a).

1.1.4.1 Menstruation and Inflammation

The process of menstruation appears to be multi-factorial and in addition to hormonal control, the process is considered to be an inflammatory event (Finn, 1986; Kelly *et al.*, 1994). Leukocytes infiltrate into the endometrium in the pre-menstrual phase, in addition to providing defence against pathogens at this vulnerable time, they are also likely to be involved directly in tissue breakdown via production of proteases, and

indirectly by the release of chemokines and cytokines. Infiltration and the roles of these different cells will be discussed in section 1.2.1. These leukocytes include macrophages, neutrophils and uNK cells and all lack genomic steroid receptors (Poropatich *et al.*, 1987; Henderson *et al.*, 2003) and therefore paracrine control over these cells must be occurring. Neutrophils are expressed across the cycle in very low numbers but rise dramatically in the immediate premenstrual phase to sites of uterine inflammation (Noyes *et al.*, 1950; Kamat *et al.*, 1987; Poropatich *et al.*, 1987). An important chemotaxis stimulus for neutrophils is IL-8 (neutrophil chemotactic factor) (Rampart *et al.*, 1989; Colditz *et al.*, 1990).

1.1.4.2 Matrix Metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are enzymes released by both the invading immune cells and by the stromal cells. The breakdown of the extracellular matrix has been attributed to the actions of MMPs (Schatz *et al.*, 1997; Lockwood *et al.*, 1998; Salamonsen *et al.*, 1999) and their role in menstruation implied by the timing of their expression within the human endometrium (Rodgers *et al.*, 1993; Hampton *et al.*, 1994; Rodgers *et al.*, 1994). MMPs are likely to be under progesterone-control since withdrawal of progesterone in cultures of endometrial stromal cells (ESCs) enhances proMMP-2 (Irwin *et al.*, 1996) and proMMP-3 (stromelysin-1) production (Schatz *et al.*, 1994). A culture system of human ESCs was used to mimic the luteal phase and demonstrated upregulation of MMP-1, -2, -3 and -4 in latent form upon progesterone withdrawal (Salamonsen *et al.*, 1997). Their tissue inhibitors (TIMPs) were also analysed and showed no regulation of TIMP-1, -2 and -3 upon withdrawal of progesterone, thus implicating a greater complexity to MMP regulation beyond that of being solely progesterone-determined. Leukocytes are another source of MMPs and via stromal and epithelial interactions, MMP release and action from this source may be being controlled (Salamonsen *et al.*, 2000).

1.1.4.3 Vascular Endothelial Growth Factor (VEGF) and Hypoxia

The type 2 receptor for vascular endothelial growth factor (VEGF), KDR, is expressed only in endometrial endothelial cells until the premenstrual phase when expression is observed in the superficial stroma (Nayak *et al.*, 2000). VEGF is expressed across the menstrual cycle (Zhang *et al.*, 1998a) and is upregulated during the menstrual phase via a hypoxic stimulus (Sharkey *et al.*, 2000). Hypoxia has been shown to raise VEGF levels in cultures of human ESCs (Popovici *et al.*, 1999) and the presence of the response element for the hypoxia inducible factor has been located on the VEGF promoter (Goldberg *et al.*, 1994). *In vitro* decidualised ESCs express greater levels of VEGF compared with controls using Microarray Technology (Popovici *et al.*, 2000). VEGF has been shown to increase the expression of MMPs (Unemori *et al.*, 1992; Wang *et al.*, 1998) and may be acting as an additional control on MMP production during the premenstrual phase (Nayak *et al.*, 2000; Critchley *et al.*, 2001b).

As discussed by Salamonsen *et al* 1999 menstrual degeneration is focal and not ubiquitous throughout the endometrium, with the regeneration and repair of the endometrium beginning to occur 36 hours after the onset of menstruation. During this repair process no scarring is evident as with normal tissue repair although the exact mechanisms are not yet understood it may be relevant in understanding pathologies such as menorrhagia (Salamonsen *et al.*, 1999).

1.1.5 Decidua and Pregnancy

Decidualisation is unique to species that undergo haemochorial placentation and in the human endometrium is considered an essential pre-requisite for establishment of pregnancy. The decidua is the transformed endometrium that is morphologically and functionally distinct. The endometrial cells differentiate and an infiltration of lymphoid cells occurs. Specific products are secreted by decidual cells, for example prolactin and IGFBP-1, and their potential functions, with regard to pregnancy, are discussed in

section 1.3.1.1. With regard to humans it is difficult to assess the direct necessity of decidualisation to implantation. However, it occurs in every menstrual cycle in advance of implantation and it may be that products of decidua are involved in this process. Conditions involving disturbance of stromal differentiation, such as endometriosis and luteal phase defect (LPD) can result in reduced fertility and menstrual irregularity (Vanrell *et al.*, 1986; Ronnberg, 1990; Lessey, 2002) and this evidence implicates decidualisation in being critical to the implantation process. LPD affects 3-4% of infertile women and can be defined as a lag of greater than 2 days histological development compared to the cycle day. This condition is believed to be due to either inadequate action or secretion progesterone, an essential stimulus for decidualisation. Decidual cells may play significant roles in controlling the extent of trophoblast invasion (Pijnenborg *et al.*, 1981). If implantation occurs in regions deficient of decidua, due to scar tissue, over-invasion occurs and conversely if under-invasion occurs the result could be the development of pre-eclampsia. Recently alpha-2 macroglobulin (α-2M), a product of endometrial endothelial cells, has been shown to be involved in decidual regulation of trophoblast invasion in mice (Esadeg *et al.*, 2003).

A 50% pregnancy loss occurs at the time of implantation and therefore it is apparent that this is a critical time for the endometrium to either continue with decidualisation or to breakdown by the process of menstruation. Decidual cells are also thought to be important in preventing uterine bleeding in the peri-implantation phase of the cycle and have been associated with spontaneous abortion and pre-term birth (Schatz *et al.*, 2001). Decidualisation initially occurs in the perivascular stromal cells and it may be that these decidual cells are involved in the control of menstruation (Kelly *et al.*, 2002). This close association with the blood vessels may allow control over the vasculature and the decision to stabilise or degenerate. These cells have been shown to produce IL-8 (Critchley *et al.*, 1994) thus stimulating neutophil influx and tissue breakdown via production of enzymes. The decision of the endometrium to breakdown or to fully differentiate is likely to be related to secretion of cytokines and other factors during implantation.

1.2 The Uterine Immune Environment

1.2.1 Leukocyte Infiltration in the Endometrium

According to the criteria of Noyes *et al* 1950, there is an infiltration of leukocytes in the late secretory phase (Noyes *et al.*, 1950) (figure 1.2). The major leukocyte populations located in human endometrium and decidua are macrophages, T cells, B cells and uNK cells and the uNK cell numbers alter in a cyclical manner (Loke *et al.*, 1997). The proliferative phase and early secretory phases have a relatively low abundance of leukocytes, as demonstrated by immunohistochemical staining with the surface marker, CD56, with an increase in the late secretory phase and into early pregnancy (Bulmer *et al.*, 1991). In addition to these CD56 positive leukocytes, an influx of neutrophils occurs in the perimenstrual period at which point they comprise approximately 6-15% of total cells, and they are thought to be important in menstruation (Salamonsen *et al.*, 1999). Eosinophils also rise immediately prior to menstruation and make up around 3-5% of endometrial cells at this point (Salamonsen *et al.*, 2000). Macrophages show a small rise in number from the proliferative phase to the secretory phase (Bonatz *et al.*, 1992) although their expression of MHC class II does not appear to vary (Bulmer *et al.*, 1988). Macrophages are also raised in number in endometrium taken from women 48 hours after controlled progesterone withdrawal compared to decidua from pregnant women (Critchley *et al.*, 1999). T and B lymphocytes are located in the basal layer in lymphoid aggregates and do not vary in number across the menstrual cycle (Bulmer *et al.*, 1991). In addition, T lymphocytes are also located throughout the functional layer in intra-epithelial locations (Loke *et al.*, 1995). These cells become activated during pregnancy at the site of large lymphoid cell clusters and it is possible they are acting to provide constitutive immune defence at this critical time or control trophoblast invasion (Mincheva-Nilsson *et al.*, 1994).

How these immune cells are regulated with regard to their recruitment in the human endometrium has not been fully established. Cells may be migrating from peripheral blood (Marzusch *et al.*, 1993) or be proliferating within the tissue itself (Klentzeris *et al.*, 1992). Endometrial chemokines may be responsible for the up-regulation of monocytes in the secretory phase (Kamat *et al.*, 1987) and high numbers of these cells are observed around the time of implantation (Bonatz *et al.*, 1992). Chemokines are chemotactic cytokines that act via G protein-coupled receptors (GPCRs) (Murphy, 1996) and their roles as chemoattractants for leukocytes will be discussed in this section. Chemokines consist of four cysteine residues and two disulphide bonds and are split into four sub-groups according to the separation of the first two cysteines by amino acids: C, CC, CXC and CXXXC (Zlotnik *et al.*, 2000).

1.2.1.2 Interleukin-8 (IL-8)

In addition to chemotaxis, IL-8 is implicated in angiogenesis (Koch *et al.*, 1992) and mitogensis of epidermal cells (Tuschil *et al.*, 1992). In the human uterus, IL-8 has been located to the perivascular cells in the late secretory phase and first trimester decidua (Critchley *et al.*, 1994; Critchley *et al.*, 1996; Critchley *et al.*, 1999; Milne *et al.*, 1999). It is also present in choriodecidual cells (Kelly *et al.*, 1992) and in amniotic fluid (Laham *et al.*, 1993). In endometriotic tissue it fails to vary with the menstrual cycle implicating persistent recruitment of leukocytes in this pathological tissue that may be under IL-8 control (Akoum *et al.*, 2001). Cultures of human ESCs and epithelial cells have demonstrated that IL-8 production can be regulated by IL-1α and TNFα (Arici *et al.*, 1993). Cell surface peptidases such as aminopeptidase N (APN) are expressed in human endometrium and their regulation of growth factors, peptide hormones and growth factors has been suggested previously (Imai *et al.*, 1992; Imai *et al.*, 1996). It has been proposed that IL-8 levels *in vivo* may be controlled via APN (Seli *et al.*, 2001). Progesterone is thought to be a main regulator of IL-8 expression. In human endometrial explant studies, progesterone acted to inhibit IL-8 secretion (Kelly *et al.*, 1994). This is in agreement with *in vivo* data that demonstrates an increase in IL-8

immunostaining in human endometrium 48 hours after progesterone withdrawal in a study designed to mimic the late secretory phase (Critchley *et al.*, 1999). This implicates IL-8 for a role in menstruation but it has also been proposed as a cervical ripening agent (Kelly *et al.*, 1992). IL-8 is able to synergise with PGE_2 to recruit neutrophils (Rampart *et al.*, 1989; Colditz *et al.*, 1990) which in turn aid the breakdown of collagen therefore softening the cervix at labour (Junqueira *et al.*, 1980).

1.2.1.3 Monocyte Chemotatic Protein-1 (MCP-1)

Monocyte chemotactic protein-1 (MCP-1) is an example of a CC chemokine and is a product of an array of cell types including fibroblasts (Yoshimura *et al.*, 1990), endothelial cells (Sica *et al.*, 1990) and monocytes (Yoshimura *et al.*, 1989) and, as its name implies, is chemotactic for monocytes (Rollins *et al.*, 1991). MCP-1 is co-expressed along with IL-8 in perivascular cells in the human endometrium in the late secretory phase (Jones *et al.*, 1997). This study also confirmed that the expression of these two chemokines coincides with COX-2 expression and implicates these factors in perimenstrual functions. Cultures of human ESCs and epithelial cells secrete MCP-1 (Arici *et al.*, 1995) and have been shown to respond to the T helper-1 cytokine, IFN-γ, by releasing MCP-1 after 24 hours of treatment (King *et al.*, 2001). Oestrogen has been shown to inhibit its secretion by ESCs (Arici *et al.*, 1999). MCP-1 production by choriodecidual cells and the breast cancer cell line, T47D, is suppressed by progesterone (Kelly *et al.*, 1997) further implicating a hormonal control over MCP-1. However, a more recent study failed to show the inhibitory effects of progesterone on either MCP-1, -2 or –3 mRNA levels in cultured human ESCs or epithelial cells (DeLoia *et al.*, 2000).

1.2.2 Uterine Natural Killer (uNK) Cells

Natural Killer cells are lymphocytes and have been shown to be critical in innate immune host defence (Bancroft, 1993; Scharton-Kersten *et al.*, 1997; Biron *et al.*, 1999; Cooper *et al.*, 2001b). They are present in peripheral blood, accounting for

approximately 10% of total blood lymphocytes (Robertson *et al.*, 1990). There is one important division with category according to their cell surface marker expression as either $CD56^{bright}$ $CD16^{-}$ or $CD56^{Dim}$ $CD16^{+}$. However, the $CD56^{bright}$ variety is scarce, comprising of less than 2% of total blood lymphocytes and approximately 10% of total blood NK cells (Lanier *et al.*, 1986). Upon activation $CD56^{bright}$ NK cells produce an array of cytokines such as IFN-γ and GM-CSF, (Cooper *et al.*, 2001a) and also develop cytotoxic activity upon IL-12 or IL-2 activation (Ellis *et al.*, 1989; Gately *et al.*, 1991; Robertson *et al.*, 1992). It has been demonstrated that the $CD56^{bright}$ NK variety produce far greater levels of cytokines compared with $CD56^{Dim}$ cells (Cooper *et al.*, 2001a).

1.2.2.1 Uterine Expression and Functions of uNK Cells

Within the uterus, NK cells were originally termed Large Granular Leukocytes (LGLs) due to their size and abundance of cytoplasmic granules (King *et al.*, 1991). They express CD56 on their cell surface making them distinct from the major blood NK cell population. However, a study comparing cytokine production by peripheral NK cells and decidual NK cells reported a very similar cytokine repertoire (Saito *et al.*, 1993). Through their secretion of cytokines it is likely they are having immunoregulatory effects. Their density is low throughout the proliferative phase but their abundance increases across the secretory phase, particularly around the time of implantation (Loke *et al.*, 1997). In the late secretory phase they comprise approximately 15–25% of the ESCs (King *et al.*, 1989b). Peripheral NK cell number is raised in women with recurrent spontaneous miscarriage of unknown aetiology, and this implies a role in controlling pregnancy maintenance (Ntrivalas *et al.*, 2001). uNK cell levels remain high into the first trimester of gestation but then decline and they also disappear in the non-pregnant endometrium prior to menstruation, possibly undergoing apoptosis (King, 2000). The first trimester appears to be a critical time for a successful pregnancy with highest miscarriage rates prevailing in this stage with approximately 50% of total human conceptions failing before the first missed menstrual period. The exact functions of these uNK cells in humans have yet to be elucidated.

How uNK cells are regulated is not yet fully understood. Their expansion across the secretory phase when progesterone levels are rising implicates progesterone as a central regulator of their growth and this is compounded by evidence that ovariectomised women lack uNK cells (Loke *et al.*, 1995). However, uNK cells lack the genomic PR and the ERα (King *et al.*, 1996), although they do express ERβ and the glucocorticoid receptor (Henderson *et al.*, 2003). This absence of the genomic PR suggests they are not controlled directly by progesterone across the secretory phase but that it is likely that regulation is via paracrine signalling with non-leukocyte ESCs since these cells persist to express the PR-A isoform at this stage of the cycle (Wang *et al.*, 1998). Studies *in vitro* have demonstrated that uNK cells proliferate upon treatment with IL-15 but other, as of yet, unidentified products of ESCs appear to increase their proliferation further (Verma *et al.*, 2000). Murine uNK cells express mRNA for an array of cytokines including CSF-1, TNF-α, IL-1, LIF and TGF-β (Croy *et al.*, 1991). A study on isolated human decidual NK cells revealed these cells express mRNA and secrete the protein for G-CSF, GM-CSF, M-CSF and LIF (Saito *et al.*, 1993). This demonstrates that a role, in addition to or in place of an immune function, exists for uNK cells although the function of these cytokines remains unclear. A close association exists between uNK cells and trophoblast cells *in vivo* providing circumstantial evidence for a role in trophoblast invasion (Loke *et al.*, 1997). In culture experiments, isolated human cytotrophoblast cells have been shown to attract $CD56^{bright}$ cells via secretion of Monocyte Inflammatory Protein 1α (MIP-1α) and implies a role for MIP-1α in attracting uNK cells (Drake *et al.*, 2001). This is in contrast to the situation in mice where those genetically-ablated for MIP-1α show no difference to wild type mice with regard to uNK cell density at the implantation site (Chantakru *et al.*, 2001). However, in mice, uNK cells are present only in the metrial gland (Croy *et al.*, 1993), the triangle at the apex of the placenta where blood vessels feeding the placenta are close together. In contrast, in humans uNK cells are present throughout the endometrium and it may therefore be necessary to "attract" these cells specifically towards the implantation site.

1.2.2.2 uNK Cells and Decidualisation

uNK cells may be linked with decidualisation. These cells were found to be specifically located in regions of stroma exhibiting pseudodecidual alterations (Bulmer *et al.*, 1988). It has been proposed that uNK cells are required in mice for maintenance of the decidual reaction rather than its initiation (Croy *et al.*, 2002). Evidence from studies on mice lacking IFN-γ implicates this cytokine, a major product of uNK cells, in the decidual reaction and conversion of the uterine vasculature (Ashkar *et al.*, 1999; Ashkar *et al.*, 2000). Rat splenocytes can be primed by prolactin to express the IL-2 receptor and therefore allows these cells to respond to IL-2 stimulation (Mukherjee *et al.*, 1990). In the human endometrium prolactin is secreted by decidualised stromal cells and mRNA expression of its receptor on these cells is under progesterone control (Tseng *et al.*, 1999). A recent study has illustrated uNK cells as a novel target for prolactin action by detection of the prolactin receptor and this may represent a functional link between uNK cells and ESCs, which produce prolactin upon decidualisation, and provide the indirect connection between progesterone control on uNK cells (Gubbay *et al.*, 2002). PGE_2 has inhibitory actions on NK cell IL-15 receptors via down-regulation of the common γ-chain (Joshi *et al.*, 2001). PGE_2 has also been shown to reduce IL-2 receptor expression on NK cells and reduce their proliferation and activity (Parhar *et al.*, 1989). Using IL-2 activated mouse uNK cells, PGE_2 stimulated an increase in the size and granularity of the cells and enhanced the proportion of 4H12 expressing cells (Linnemeyer *et al.*, 1993). The uNK cells accumulating at implantation sites immuno-stain for the 4H12 antibody and they were able to demonstrate in culture that these cells were less cytotoxic than NK cells negative for 4H12. The described effects of PGE_2 are likely to be mediated via cAMP since this second messenger was able to augment the same responses.

Angiogenesis is a critical process in the human endometrium and ensures that vascular remodelling of the spiral arterioles and regeneration of the vasculature following menstruation can take place. A major angiogenic stimulus is VEGF and it is a member

of the vascular endothelial growth factor family, of which there are six members. VEGF protein and mRNA have been localised within the endometrium across all phases of the menstrual cycle in both the glandular epithelial cells and in the stroma (Charnock-Jones *et al.,* 1993). In cultures of human ESCs progesterone treatment resulted in a rise in VEGF, isoform $VEGF_{189}$, secretion (Ancelin *et al.,* 2001). Hypoxia and cAMP have also been identified as stimuli for VEGF production in ESCs *in vitro* as demonstrated by ELISAs and Northern Blot analysis (Popovici *et al.,* 1999). uNK cells are present in the stroma across the menstrual cycle and increase in number across the secretory phase and into the first trimester of gestation (Loke and King, 1997). Across the secretory phase uNK cells have been shown to express VEGF-C and placenta growth factor (PIGF) mRNA (Li *et al.,* 2001). Additionally, in the late secretoy phase uNK cells express mRNA for the angiopoietin, Ang2. This study also demonstrated the ability of IL-15 to up-regulate VEGF-C mRNA levels in uNK cells. The specific locality of uNK cells within the stroma is in close proximity to the glands and the blood vessels and therefore their production of angiogenic factors may be having a direct influence on the blood vessels. This supports a possible involvement in menstruation or in the stabilisation of blood vessels during implantation and early pregnancy (King and Loke, 1990).

1.2.3 Glandular Cells in Immune and Reproductive Functions

The epithelium is considered central in intestinal immune function (Gewirtz *et al.*, 2002) and in the control of lymphocyte trafficking in, for example, the skin and small intestine (Kunkel *et al.*, 2002). Within the endometrium the glands are composed of epithelial cells and they undergo cyclical variation as described in sections 1.1.1 and 1.1.2. Figure 1.3 illustrates the structure of a gland in the early secretory phase. Infiltrating leukocytes in the mid and late secretory phase are situated immediately adjacent to glands in the human endometrium (Bulmer *et al.*, 1985). Macrophages send long processes into the epithelium of the glands of human endometrium (Kamat *et al.*, 1987) and intraepithelial T-lymphocytes have been identified (Loke *et al.*, 1995). The glandular cells are also HLA-DR-positive in the secretory phase but not in the

proliferative phase (Chiang *et al.*, 1997) and an increased accumulation of extracellular immunoglobulin is apparent within glands with progression of the menstrual cycle (Bjercke *et al.*, 1993) implying a specific immune function in secretory endometrium. Lymphocytes have been characterised in early human decidua and located as either clusters, forming aggregates, near to the glands or as intraepithelial lymphocytes within the glands themselves (Mincheva-Nilsson *et al.*, 1994).

In addition to the leukocyte populations, endometrial epithelial cells are believed to be important in host defence. Defensins are small cationic proteins that confer anti-bacterial, anti-fungal and anti-viral properties and human beta-defensins 1-4 (HBD1-4) mRNA is expressed in the human endometrium (Valore *et al.*, 1998; Fleming *et al.*, 2003; King *et al.*, 2003). Secretory leukocyte protease inhibitor (SLPI) is also a product of human endometrial epithelial cells (King *et al.*, 2000) and has been reported to have antibacterial, antiviral and antifungal effects (Tomee *et al.*, 1998). Both HBD-1 and SLPI increase in the mid secretory phase although SLPI expression rises further in the late secretory phase and into pregnancy (King *et al.*, 2000; Fleming *et al.*, 2003). They may have important functions at the time of implantation and in early pregnancy but since the glands lack the progesterone receptor at this time, stromal-derived factors may be important in controlling their production since the stroma in the functional layer retains the PR-A receptor (Wang *et al.*, 1998).

The COX enzymes are involved in regulation of PGE_2 synthesis and are discussed in section 1.4.1. Both isoforms have been located in the human endometrium with COX-1 expression being predominant in the luminal and glandular epithelial cells (Rees *et al.*, 1982) and COX-2 being expressed in the glands and perivascular cells in the luteal phase (Jones *et al.*, 1997; Marions *et al.*, 1999). Treatment with the antiprogestin, RU486, in the luteal phase reduced the expression of both isoforms in the epithelial cells and a role in endometrial receptivity was proposed (Marions *et al.*, 1999). Studies on mice knock-outs for the COX enzymes have implicated COX-2 in ovulation, fertilisation, implantation and decidualisation (Lim *et al.*, 1997). In the COX-1 knock-

out it has been proposed that COX-2 is compensating for the COX-1 deficiency (Reese *et al.*, 1999).

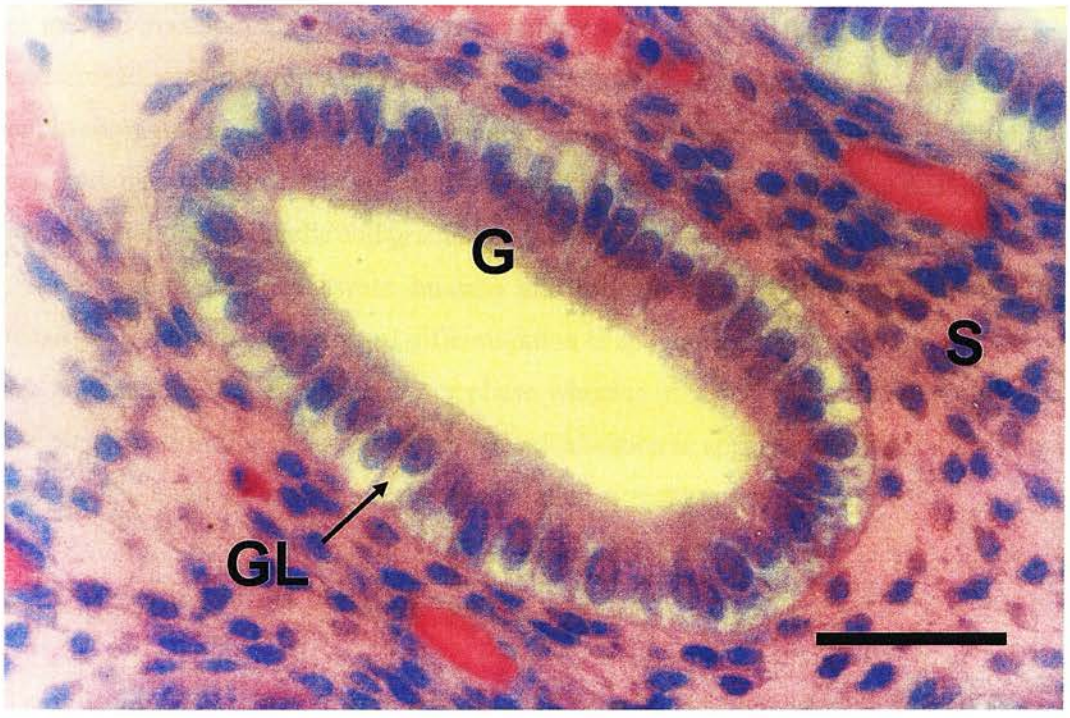


Figure 1.3

Photomicrograph of a gland in the early secretory phase endometrium at high power magnification in human endometrium stained with toluidine blue and acid fuschsin. **G** = gland; **S** = stroma; **GL** = glycogen deposits. Scale bar represents 40μm.

1.3 Establishment of Pregnancy

1.3.1 Decidualisation

The transformation of the human endometrium into decidua begins around post-ovulatory day 9 in oestrogen-primed stromal cells surrounding the spiral arterioles. This reaction then spreads throughout the stroma completing the pre-decidual transformation of the endometrium. If pregnancy is established, these decidual changes become more widespread creating three layers: decidua compacta, decidua spongiosa and a basal undifferentiated layer, allowing regeneration following menstruation or birth. Studies in rats and mice identified three types of decidual cell: anti-mesometrial decidual cells, mesometrial decidual cells and granulated metrial gland cells (De Feo, 1967). However, decidualisation differs between humans and rodents with regard to its stimulus and locality. In humans, the stromal differentiation is spontaneous and occurs in preparation for implantation across the secretory phase whereas in rodents it is initiated only upon the physical stimulus of implantation. Decidualisation is apparent throughout the human endometrium but only occurs at the implantation site(s) in mice and rats.

1.3.1.1 Decidualisation Markers

Term decidual fibroblasts from humans have been shown to express desmin (Oliver *et al.*, 1999). In addition, decidualised ESCs express raised levels of fibronectin and laminin compared with control cultures (Irwin *et al.*, 1989). Rat decidual cells contain large quantities of intermediate fibres. These are made up of vimentin, which is also present in non-decidualised stromal cells, and desmin, specific to decidualised cells (Glasser *et al.*, 1986). However, two other products of the decidual reaction, prolactin and IGFBP-1, are more commonly used to mark this process in culture experiments using human ESCs. In addition to this a further marker of this process is tissue factor and these will be discussed each in turn.

Prolactin is an anterior pituitary hormone, but was first detected being produced outside the pituitary, by decidualised endometrial cells (Riddick *et al.*, 1978). It was then confirmed that prolactin synthesis correlated with the extent of stromal decidualisation in a study of human endometrial explants from the various stages of the menstrual cycle (Maslar *et al.*, 1979). Prolactin is widely expressed and has been located in dermal fibroblasts, epithelial cells of the prostate and breast, Leydig cells of the testis and by certain immune cells as summarised in Ben-Jonathan *et al* 1996 (Ben-Jonathan *et al.*, 1996). Mouse gene knock-outs have confirmed that prolactin is critical to reproductive processes and fertility and particularly with regard to implantation and pregnancy maintenance (Binart *et al.*, 2000). Within the endometrium, prolactin protein is expressed primarily in stromal cells in the late secretory phase but epithelial cells were also immunoreactive for prolactin at this point in the cycle (Bryant-Greenwood *et al.*, 1993). Whilst the protein has also been confirmed in both stromal and epithelial cells, mRNA has only been detected in the stromal cells (Dimitriadis *et al.*, 2000). Prolactin is considered to be important in human implantation and early pregnancy, as discussed in Jabbour and Critchley 2001 (Jabbour *et al.*, 2001). A novel target cell of prolactin has been identified as uNK cells since these cells express the prolactin receptor (Gubbay *et al.*, 2002). Prolactin has also been shown to regulate the expression of interferon-regulatory factor (IRF-1), which is located in the glands and upregulated in the secretory phase (Jabbour *et al.*, 1999).

The insulin-like growth factor system consists of peptide growth factors (IGFs) and their receptors but also specific binding proteins (IGFBPs) that regulate the availability of IGFs to their receptors (Lamson *et al.*, 1991). Six types of soluble IGFBPs exist and these have a degree of tissue specificity (Shimasaki *et al.*, 1991). Within the human endometrium, mRNA for all six IGFBPs were located and with the exception of IGFBP-5, all increased in the secretory phase compared with the proliferative stage (Zhou *et al.*, 1994). The production of IGFBP-2 and -3 by cultures of ESCs with oestrogen and progesterone treatment has been demonstrated (Giudice *et al.*, 1991). Immunohistochemical studies have demonstrated an increase in IGFBP-1 levels in

human endometrium in the secretory phase (Bryant-Greenwood *et al.*, 1993) and IGFBP-1 was found to be a secretory product of secretory phase endometrium and early decidua (Bell *et al.*, 1989). IGFBP-1 is considered a marker of decidualisation and is used to assess this process in cultures of ESCs (Irwin *et al.*, 1989; Bell *et al.*, 1991; Giudice *et al.*, 1992). The specific function of IGFBP-1 in the uterus in unknown although it may be regulating IGF-1, a stimulator of cell growth, and IGF-1 mRNA levels are up-regulated in the rat uterus following oestrogen treatment (Murphy *et al.*, 1987). In women using LNG-IUS mRNA levels of IGFBP-1 are upregulated, consistent with immunohistochemical stromal staining, and IGF-1 levels were found to be decreased (Rutanen, 2000). It was speculated that the inhibitory action of IGFBP-1 on IGF-1 levels could partially account for the progestogenic and anti-oestrogenic effects of LNG-IUS.

Tissue factor is a membrane-bound glycoprotein. It has an extracellular domain that acts as a receptor for factor VII that is involved in the thrombin pathway and it thought to maintain an equilibrium between haemostatic and fibrinolytic pathways as discussed in Schatz *et al* 2001(Schatz *et al.*, 2001). This knowledge, along with *in utero* haemorrhage of tissue factor knockout mice, (Carmeliet *et al.*, 1996) implies a role for tissue factor in regulation of bleeding. In women using Norplant, the observed decline in endometrial tissue factor protein and mRNA levels may partially account for their irregular bleeding patterns (Runic *et al.*, 1997). Tissue factor has also been implicated in angiogenesis via induction of VEGF (Abe *et al.*, 1999). Experiments on human ESCs in culture have confirmed hormonal control over the increase in tissue factor expression and withdrawal of oestrogen and progesterone resulted in a return of tissue factor levels to those similar to pre-treatment levels (Lockwood *et al.*, 1993). Cyclical changes in tissue factor mRNA and protein are observed in humans with greatest levels of expression in luteal phase stroma and in decidua (Lockwood *et al.*, 2001). Its presence may be important in having a primary stabilisation function during the destructive process of trophoblast invasion and paralleled vascular remodelling (Schatz *et al.*, 2001).

1.3.1.2 Progesterone and cAMP

Progestins stimulate the production of prolactin and its receptor in stromal cell primary cultures and this can be inhibited by addition of the anti-progestin, RU486 (Tseng *et al.*, 1999). Studies on ESC decidualisation *in vitro* have demonstrated that progesterone treatment alone is a weak inducer of the decidual transformation and can only achieve this conversion after prolonged exposure (Huang *et al.*, 1987; Zhu *et al.*, 1990; Tseng *et al.*, 1992; Mizuno *et al.*, 1998). It has also been reported that women with low serum progesterone levels at four weeks gestation can have successful pregnancies following assisted reproductive technologies (ART) treatment (Azuma *et al.*, 1993) and it may be the case that other factors are important, in addition to progesterone, to decidualisation. Synthetic progestins, such as medroxyprogesterone acetate (MPA), are usually used in place of progesterone because they are metabolically stable and thus more effective at inducing prolactin expression (Zhu *et al.*, 1990). However, addition of cAMP in combination with progesterone produces this conversion on a greatly reduced time scale (Tang *et al.*, 1993a; Tang *et al.*, 1993b; Brar *et al.*, 1997) and these actions are probably via the protein kinase A pathway (Brar *et al.*, 1997).

1.3.1.3 Relaxin

Relaxin is similar in structure to insulin and insulin-like growth factors (Blundell *et al.*, 1980) and consists of two chains, A and B chains, which are covalently linked by two disulphide bonds (Schwabe *et al.*, 1978; Schwabe *et al.*, 1978). It is synthesised as a precursor form, preprorelaxin (Kemp *et al.*, 1984). Relaxin is primarily a product of the corpus luteum in reproductive tissue but has been isolasted from human decidua by chromatographic elution (Bigazzi *et al.*, 1980). It has been localised to the glands and stroma of human endometrium and decidua, as shown by immunohistochemistry (Bryant-Greenwood *et al.*, 1993). Relaxin is secreted by the ovary and is present in the blood and therefore some of the immunoreactivity for relaxin in the endometrium may be from this source. However, if the sole source of relaxin in this tissue was from

outside of the endometrium it may be expected that highest concentrations of relaxin immunoreactivity would be observed surrounding the blood vessels. In fact, highest immunoreactivity intensity is found in the glandular cells. In addition, two molecular forms of relaxin exist, H1 and H2 and are encoded by different genes (Hudson *et al.*, 1983; Hudson *et al.*, 1984). H2 is the main form produced by the ovary whereas H1 and H2 are produced by decidua (Bryant-Greenwood, 1991; Hansell *et al.*, 1991). Within the stroma, relaxin was immunolocalised in decidualised cells in the late secretory phase and throughout early and late gestation. ESCs themselves have very few receptors for relaxin, approximately 1000 per cell (Osheroff *et al.*, 1995), however, in cultures of human ESCs relaxin both acutely and permanently elevates intracellular cAMP levels in concert with induction of prolactin secretion (Telgmann *et al.*, 1998) and produces maximal stimulus on prolactin production when added in combination with a synthetic progestin (Huang *et al.*, 1987; Zhu *et al.*, 1990). In these early studies porcine relaxin was used in the treatment of human ESCs. Whether this is an exact mimic of human relaxin actions has not been confirmed due to a lack of availability. However, relaxin does have the effect of raising cAMP and the direct application of cAMP to cultures results in prolactin secretion by human ESCs in a similar fashion (Tang *et al.*, 1993a; Tang *et al.*, 1993b; Brar *et al.*, 1997). It has further been demonstrated that the induction of raised cAMP levels by relaxin treatment is due to its action to inhibit phosphodiesterase activity and therefore inhibit cAMP destruction (Bartsch *et al.*, 2001).

1.3.1.4 Interleukin-11 (IL-11)

IL-11 is a member of the gp130 cytokine family along with LIF (Gadient *et al.*, 1999). It has actions in a wide range of environments from haematopoietic cells to the nervous system (Hawley, 1994) but also has anti-inflammatory actions in the gastrointestinal tract (Sands *et al.*, 1999). Within the human endometrium IL-11 is expressed in all cell types with greatest immunostaining intensities apparent in decidualised stroma and appears in these cells before prolactin (Dimitriadis *et al.*, 2000). Human ESCs produce IL-11 and express IL-11 receptor α (IL-11Rα) in culture during progesterone-induced

decidualisation and upon treatment with an anti-human IL-11 antibody a reduction in prolactin and IGFBP-1 levels were observed implicating IL-11 in induction of decidualisation (Dimitriadis *et al.*, 2002). Production of IL-11 by cultured human epithelial cells and stromal cells was enhanced by IL-1α, TNF-α and TGF-β and these results suggest a role for IL-11 in implantation (Cork *et al.*, 2001). In the murine uterus IL-11 is essential to female fertility as shown by IL-11Rα gene knockout studies in which a defective uterine response to blastocyst fails to trigger normal maternal decidual transformation (Bilinski *et al.*, 1998; Robb *et al.*, 1998).

LIF may also have a role in the process of decidualisation since LIF null mice fail to exhibit decidualisation (Stewart *et al.*, 1992b). The reproductive roles of LIF are discussed further in section 1.3.2.2. Recently, mRNA and protein for ghrelin, a peptide hormone, have been shown to increase in human decidualised stroma and in addition, enhanced cAMP-induced decidualisation of cultured human ESCs (Tanaka *et al.*, 2003). The mechanisms and factors involved in decidualisation are complex and it is likely that other factors that are involved have not yet been identified.

1.3.1.5 Decidualisation and Cell Growth

Decidualisation treatments affect the growth rate of ESCs. For example, MPA stimulates cell growth and prolactin production in human ESCs in culture whereas relaxin does not promote cell growth (Zhu *et al.*, 1990). IL-11 increases tritiated thymidine uptake by human ESCs in culture (Karpovich *et al.*, 2003). In rat ESCs both progesterone and prostaglandins have been postulated to exert effects on the rate of DNA synthesis with progesterone stimulating it and PGE_2 reducing it (Peleg, 1983). This is consistent with the finding that in rats treated with RU486, division of stromal cells during pregnancy was inhibited compared with untreated controls (Rider *et al.*, 1994). The proliferative effects of basic fibroblast growth factor (bFGF) are dependent on the presence of progesterone in cultures of human ESCs (Irwin *et al.*, 1991). In addition to this the insulin-like growth factor (IGF) system has been directly implicated

in human ESC decidualisation (Irwin *et al.*, 1994). This study demonstrated the necessity of IGF plus epidermal growth factor (EGF) in combination for promotion of cellular proliferation. However, the presence of growth factors was not essential to stimulate prolactin and IGFBP-1 production. It has been shown that term decidua contains undifferentiated ESCs that can be stimulated *in vitro* to decidualise and it is speculated that these represent a proliferating population of ESCs that are maintained throughout pregnancy but may be recruited to decidualise during gestation (Richards *et al.*, 1995).

1.3.2 Implantation

The main influences on the sequential maturation of the endometrium are oestrogen and progesterone with the latter being dominant in the luteal phase of the cycle. Imbalances of these hormones and/or their receptors can result in an out-of-phase development that may be significantly detrimental to the implantation process (Bonhoff *et al.*, 1990). Studies in rodents demonstrated a maternal implantation window controlled by the steroid hormones (Psychoyos, 1986). In humans, implantation occurs around day LH + 6 and LH + 7 (Hertig *et al.*, 1956; Bergh *et al.*, 1992) and the concept of the "implantation window" specifies that the blastocyst can only implant whilst the endometrium is in a receptive developmental stage (Navot *et al.*, 1989; Li *et al.*, 1991; Tabibzadeh, 1998). The processes of implantation and decidualisation are likely to be closely linked, although in humans decidualisation begins prior to implantation on day LH + 8. However, these two events are likely to be closely coordinated and one important feature of a receptive endometrium is decidualisation. In a review of oocyte donation success, the optimal window for transferring the fertilized oocyte was between cycle days 16 and 19 (Rosenwaks, 1987). After this period, no pregnancies were established. Ultrastructual changes in the luminal epithelial cells are also thought to be correlated with this receptivity status (Sarani *et al.*, 1999). However, implantation has been shown to be dependent on embryonic age rather than endometrial maturation in another study involving IVF treatment of women (Bergh *et al.*, 1992). The receptive endometrium is thought to balance implantation with excessive trophoblast invasion by providing appropriate signals (Tabibzadeh *et al.*, 1995) and embryonic signalling may be essential for the implantation process and maternal acceptance (Edwards, 1994).

The implantation process itself is quite unique in humans in comparison to other species and is haemochorial (Pijnenborg *et al.*, 1981). The first stage of implantation is fertilisation of the oocyte within the fallopian tube. The fertilized oocyte then undergoes successive cleavages during the week-long peri-implantation period. These cleavages must generate sufficient cell numbers prior to blastocyst formation to allow completion

of inner cell mass and trophectoderm formation and are followed by proliferation and migration towards the uterine cavity around 72–96 hours post fertilisation. On day 5, the blastocyst hatches from the zona pellucida. The actual process of implantation occurs in three stages, the first of these being apposition. This is followed by adhesion of the blastocyst to the luminal epithelium via the trophectoderm and allows the trophoblast to burrow between the luminal epithelial cells, leaving these luminal epithelial cells undisturbed. Placenta formation starts once the trophectoderm has invaded and become embedded in the decidua. Some of the cytotrophoblast cells then extend into the peripheral syncytium and join to form the cytotrophoblastic shell. Villous trophoblast cells will eventually form a layer over the placenta and have the function of nutrient and oxygen transport from maternal blood to the fetus. The extravillous trophoblast cells penetrate deep into the decidua and transform the spiral arteries, destroying the muscular walls and converting them into larger vessels allowing delivery of greater volumes of blood. This is essential for an adequate blood supply for the fetus and in cases of under-invasion of the trophoblast pathologies such as miscarriage, pre-eclampsia and fetal intrauterine growth restriction can result (Loke *et al.*, 1995). This is not only relevant to the short-term with regard to pregnancy complications but can lead to the development of problems in adult life (Barker, 1997b). Conversely, when over-invasion of the trophoblast occurs a phenomenon of placenta percreta, or uterine rupture, can develop. Therefore, it is important for this process to be under stringent control and a synchrony between the blastocyst and endometrium is required for it to be implemented successfully.

1.3.2.1 Colony Stimulating Factor-1 (CSF-1)

Signalling between the blastocyst and the endometrium are likely to be essential prior to adhesion to the epithelium and several soluble factors have been implicated. Colony-stimulating-factor-1 (CSF-1) is a growth factor and is expressed within the endometrium in the stroma and epithelial cell and also by the trophoblast along with its receptor, c-fms (Daiter *et al.*, 1992). Mutant mice lacking CSF-1 are infertile due to implantation failure

(Pollard *et al.*, 1991). The cyclical variation of CSF-1 and c-fms in humans is characterised by rapid rises in expression in the late secretory phase and early pregnancy (Kauma *et al.*, 1991).

1.3.2.2 Leukaemia Inhibitory Factor (LIF)

LIF is a glycoprotein expressed in endometrial glands just prior to implantation in mice (Bhatt *et al.*, 1991) and may be involved in blastocyst growth and implantation (Bulletti *et al.*, 1994; Polan *et al.*, 1995). A targeted mutation of the maternal LIF gene resulted in impaired implantation in mice. The blastocysts however were viable and able to implant in wild-type pseudo-pregnant mice implicating LIF specifically in implantation (Stewart *et al.*, 1992b). In contrast, LIF is not critical to implantation in sheep and cows as determined by experiments in which animals were immunised against LIF (Vogiagis *et al.*, 1997). In humans, LIF is present within endometrium and at highest levels during the time of implantation (Charnock-Jones *et al.*, 1994; Kojima *et al.*, 1994). Isolated first trimester trophoblast is negative for LIF but positive for its receptor (King *et al.*, 1995) and it could be speculated that endometrial LIF is acting on its receptors located on trophoblast cells. Some of the actions of LIF may be via human chorionic gonadotrophin (hCG) since anti-hCG antibodies have been shown to block LIF-mediated trophoblast differentiation (Sawai *et al.*, 1995). hCG is secreted by the embryo and by preventing demise of the CL and therefore maintains luteal progesterone production. The actions of hCG may not be confined to the CL since hCG receptors have also been located in the human endometrium (Reshef *et al.*, 1990) and in support of this, human endometrial stromal cells treated with hCG decidualise (Han *et al.*, 1999).

1.3.2.3 Cell-matrix Interactions

Interactions between the extracellular matrix and cells provide an essential communication route. The interactions between extracellular matrix constituents and the surface of cells are predominantly mediated by integrins. Integrins are a family of cell surface heterodimeric α/β glycoproteins and are able to bind matrix molecules and to cell surface receptors (Hynes, 1992). They participate in adhesion, migration, proliferation, differentiation and cell survival. Several integrins are expressed within the human endometrium (Lessey *et al.*, 1992; Tabibzadeh, 1992). The expression of many of these fluctuates in a cyclical manner and those with expression confined to the window of implantation are $\alpha_1\beta_1$, $\alpha_4\beta_1$ and $\alpha_v\beta_3$ (Tabibzadeh, 1992; Lessey *et al.*, 1994a). Expression of the fibronectin receptor is delayed in luteal phase defect (Lessey *et al.*, 1992) and aberrant expression of this receptor has also been associated with endometriosis (Lessey *et al.*, 1994b).

Matrix metalloproteinases (MMPs) are enzymes that have a range of specificity for extracellular matrix molecules and are invloved in their degradation. MMPs are generally considered pathological when detected in connective tissue, however, they have been localised in human trophoblast (Polette *et al.*, 1994) and are considered to have a role in the uterus of physiological tissue remodelling (Hulboy *et al.*, 1997). Tissue inhibitor of MMPs (TIMP) inhibits the invasiveness of trophoblastic cells *in situ* (Graham *et al.*, 1991). MMP2 and MMP9 are members of the gelatinase subfamily and are secreted by the invasive human trophoblast (Shimonovitz *et al.*, 1994). They act to digest collagen type IV, the major constituent of the uterine basement membrane, and would be important in uterine invasion during implantation. MMPs are discussed in more depth in section 1.1.4.2.

The interactions between leukocytes and ESCs via the production of cytokines and growth factors, such as VEGF, the interferons and TNF-α, are believed to be important in endometrial tissue breakdown and remodelling (Fraser, 1999). These immune cells

are believed to play a significant role in establishment of microenvironments via their production of such factors (Tabibzadeh *et al.,* 1993; King, 2000). uNK cells are located in the stroma in high concentrations around the blood vessels and are a source of VEGF and their potential effects on the blood vessels are briefly assessed in section 1.2.2.2. In addition, uNK cells are also located at the implantation site and this is their only location within the mouse endometrium (Croy *et al.,* 1991). They may be involved in modulating trophoblast invasion in humans (King and Loke, 1990) and in mice have been proposed as having a role in the regulation of placental development whereby knockout mice for uNK cells exhibit reduced placental size (Croy *et al.,* 2002). However, a more crtical role in mice is the stabilisation of the vasculature and complete differentiation of the endometrium into decidua (Ashkar *et al.,* 2000). IFN-γ-producing immune cells, in particular uNK cells, are thought to be involved in modulating ESC decidualisation in humans (Christian *et al.,* 2001). They demonstrated the ability of IFN-γ to antagonise prolactin protein and mRNA expression in primary ESC cultures. This provides a role for IFN- γ as a modifier of ESC function.

1.3.3 The Role of Cytokines

Cytokines are small glycoproteins with autocrine and paracrine interactions and are predominantly associated with immune functions. They can be involved in both the induction and resolution of an inflammatory response and can generally be termed "pro-inflammatory" or "anti-inflammatory". Studies on CD4+ T-cells showed that responses of these cells are polarised and they have been classified on their cytokine production as either T-helper-1 (Th-1) or T-helper-2 (Th-2) (Mosmann *et al.*, 1986). The Th-1 cytokines include IFN-γ, IL-2 and TNFα and whilst examples of Th-2 cytokines are IL-4, IL-10 and IL-13. In the human endometrium during the peri-implantation phase, a reduction or absence of Th-1 and a dominance of Th-2 cytokines is observed (Lim *et al.*, 1998). The general hypothesis with regard to pregnancy that has been derived from mice studies is that a Th-1 response is considered detrimental and a Th-2 response is thought to be beneficial and supportive of pregnancy and the balance of these responses

is likely to be critical (Wegmann *et al.*, 1993; Hill *et al.*, 2000), although it is now apparent that this is an over-simplification (Chaouat *et al.*, 2002). Some of the reproductive functions of cytokines are summarised in table 1.2.

Cytokine	Endometrial function
IL-1	Induction of adhesion molecules Modulation of proliferation Alteration of morphology Induction of cytokines Chemotaxis and induction of lymphoid infiltration Oedema Induction of PGE_2 Activation of T cells
TGF-β	Gland formation and Angiogenesis
IFN-γ	Induction of adhesion molecules Modulation of proliferation Alteration of morphology Induction of cytokines Chemotaxis and induction of lymphoid infiltration Induction of menstruation Induction of HLA-DR and ICAM-1
TNF-α	Induction of adhesion molecules Modulation of proliferation Alteration of morphology Induction of cytokines Chemotaxis and induction of lymphoid infiltration Oedema Induction of PGE_2 Injury to endometrial vessels Activation of polymorphonuclear leukocytes

Table 1.2

Cytokine functions in the human endometrium. Table adapted from Tabibzadeh 1994 (Tabibzadeh, 1994a).

This section will examine IL-1, IL-10 and IFN-γ and their functions within the uterus. IL-8 and IL-15 are discussed in more detail in sections 1.2.1.2 and 1.4.3 respectively.

1.3.3.1 Interleukin-1 (IL-1)

IL-1 is a multifunctional cytokine and exists in two forms: IL-1α and IL-1β with a low sequence homology between them. They have similar action and act on the same receptor, IL-1 type-1. A second receptor sub-type exists, IL-1 type-2, but it is non-functional. In humans, the IL-1 receptor antagonist is located in decidual glands, and in isolated cells in chorionic villi, the intervillous space and in maternal decidua (Simon *et al.*, 1994). These decidual cells staining positive for the IL-1 receptor antagonist are thought to be macrophages (Tabibzadeh *et al.*, 1992). IL-1β is the dominant form and is secreted by activated murine peritoneal macrophages (Chensue *et al.*, 1989). In the human endometrium both IL-1α and IL-β are expressed in the epithelial and stromal cells (Tabibzadeh *et al.*, 1992) and IL-β mRNA expression is raised in pregnancy (Kauma *et al.*, 1990). Human serum IL-1 levels vary according to the menstrual cycle and are found to be highest in the secretory phase, post-ovulation (Cannon *et al.*, 1985). In contrast, IL-1 receptor antagonist levels decline in the secretory phase (Simon *et al.*, 1995). IL-1 has also been connected to blastocyst implantation and is found expressed along with its receptor in both trophoblast and decidual cells (Simon *et al.*, 1994; Simon *et al.*, 1995). ESCs produce an immune-related response upon treatment with IL-1α by evoking an enhancement in phagocytosis of latex particles and E.coli (Ruiz *et al.*, 1997). This cytokine may also be involved in menstruation since in cultures of human ESCs, IL-1α stimulates production of MMP-1, a key enzyme in menstruation initiation (Singer *et al.*, 1997). IL-1β acts to raise PGE_2 levels in decidual and endometrial ESCs (Cole *et al.*, 1995; Ishihara *et al.*, 1995). IL-1α stimulates PGE_2 and $PGF_{2\alpha}$ production in ESCs (Kawano *et al.*, 2001). These data support a role for IL-1 in the regulation of menstruation since PGE_2 and $PGF_{2\alpha}$ are believed to be involved in vascular changes associated with endometrial tissue degradation (Baird *et al.*, 1996). IL-1 has also been

associated with endometriosis. Women with endometriosis have elevated IL-1β in their perintoneal fluid (Fakih *et al.*, 1987) although a later study determined raised IL-1 receptor antagonist in association with endometriosis as opposed to IL-1β (Mori *et al.*, 1992). IL-1 has been demonstrated to activate an angiogenic phenotype in human ESCs from endometriotic lesions via its receptor (Lebovic *et al.*, 2000).

1.3.3.2 Interleukin-10 (IL-10)

IL-10 is a homodimeric cytokine expressed by naive and memory T cells (Chabot *et al.*, 1999). In blood NK cells, IL-2 alone or synergistically with IL-12, can act to induce IL-10 expression (Mehrotra *et al.*, 1998) and this has also been demonstrated in uNK cells (Vigano *et al.*, 2001). In mice, IL-10 is only expressed by Th-2 cells but in contrast this cytokine is expressed by both Th-1 and Th-2 cells although the latter express it more strongly (Romagnani, 2001). IL-2-induced $CD56^{bright}$ NK cells proliferate and produce cytokines upon treatment with IL-10 (Carson *et al.*, 1995). Within the pregnant uterus, trophoblastic villi do not secrete IL-10 (Kelly *et al.*, 1995), however, mononuclear cells in the decidua do so spontaneously in culture (Ekerfelt *et al.*, 2002). The specific roles of IL-10 in reproduction are unclear. Double-deficient mice for IL-10 and IL-4 have successful pregnancies, although this study did not assess fetal or maternal development (Svensson *et al.*, 2001). A more recent study on IL-10 null mice has demonstrated abnormal placental development in the absence of IL-10 and has suggested a role for this cytokine in implantation and fetal programming (Roberts *et al.*, 2003). In women with missed abortion, a reduction of IL-10 immunostaining was observed in uNK cells compared with those from elective terminations (Plevyak *et al.*, 2002). These results may actually represent post-miscarriage inflammatory changes and it is therefore difficult to rely on this data alone. In contrast to this, IL-10 production was greater in stimulated peripheral blood mononuclear cells from women with recurrent pregnancy loss than controls (Bates *et al.*, 2002). This may, however, represent a compensatory mechanism by which IL-10 counteracts other not yet understood malfunctions. This

study was also assessing NK cells from blood and whether they have any impact on uNK cells has not been confirmed.

1.3.3.3 Interferon-γ (IFN-γ)

The interferon family are cytokine mediators that are involved in alterting the immune system to the presence of viral infections. The family comprises of three members in humans: IFN-α, -β and -γ. Components of the same receptor are shared by IFN-α and IFN-β and they are therefore referred to as type I interferons. Since IFN-γ uses a separate receptor system it is termed a type II interferon. In addition to distinct receptors, type I and II interferons also differ with regard to which chromosome they are located on and their structure differs. IFN-γ is distinct due to its sensitivity to pH and temperature extremes (Arai *et al.*, 1990) and is expressed by many cell types including Natural Killer cells and macrophages, but predominantly by T cells (Sugawara *et al.*, 1986). In contrast, type I interferons are secreted by virus-infected cells (Le Page *et al.*, 2000).

IFN-τ is a member of the interferon I family. It is not present in humans but is expressed by ruminant trophoblastic cells although it has structural and biological functions similar to those of IFN-α and -β, both present in humans (Roberts *et al.*, 1999). IFN-τ is believed to be the maternal recognition of pregnancy signals in ruminants. IFN-τ is also secreted by the bovine conceptus and is considered to be involved in rescue of the corpus luteum via limitation of $PGF_{2\alpha}$ release (Thatcher *et al.*, 1995).

The source of IFN-γ within the human endometrium is thought to be the lymphoid aggregates (Tabibzadeh, 1994b) but is also synthesised by uNK cells in first trimester decidua (Saito *et al.*, 1993) and by macrophages (Gessani and Belardelli, 1998). IL-12 can stimulate IFN-γ production in cultures of uNK cells and acts synergistically with IL-

2 to provide this response (Marzusch *et al.*, 1997). IFN-γ levels remain consistent across the menstrual cycle in non-pregnant endometrium (Yeaman *et al.*, 1998) but during pregnancy it can only be detected in supernatants from first trimester decidua and not in the later pregnancy stages (Lin *et al.*, 1993). IFN-γ acts on the epithelial cells to induce human leukocyte antigen (HLA)-DR molecules of MHC and triggers morphological changes in addition to a reduction in growth (Tabibzadeh *et al.*, 1986; Tabibzadeh *et al.*, 1988). This cytokine also has actions on ESCs to induce an increase in the levels of IL-6, MCP-1 and MCSF production and reduce IL-8 levels (Nasu *et al.*, 1998).

Table 1.3

Summary of the reproductive phenotypes exhibited by mutated mice genetical deficient in endometrial cytokines or their receptor. This table is adapted fro Robertson and Hudson 2002 (Robertson *et al.*, 2002).

Cytokine	Mutated reproductive phenotype	Reference
CSF-1	Macrophage deficiency Gametogenesis dysregulation	Pollard *et al* 1991 (Pollard *et al.*, 1991)
GM-CSF	Increased fetal reabsorption Placental abnormalities	Seymour *et al* 1997 (Seymour *et al.*, 1997) Robertson *et al* 1999 (Robertson *et al.*, 1999)
IFN-γ	Dysregulation of uNK cells Decidual necrosis Inadequate transformation of endometrial vessels during decidualisation Increased fetal reabsorption	Ashkar and Croy 1999 (Ashkar *et al.*, 1999)
IL-1R	Reduced litter size	Abbondanzo *et al* 1996 (Abbondanzo *et al.*, 1996)
IL-5	Eosinophil deficiency Apparently uncomplicated pregnancy	Robertson *et al* 2000 (Robertson *et al.*, 2000)
IL-11Rα	Defective decidualisation Failed implantation	Robb *et al* 1998 (Robb *et al.*, 1998)
IL-2 Rγ	Fail to develop uNK cells	Miyazaki *et al* 2002 (Miyazaki *et al.*, 2002)
LIF and its R	Failed implantation Placental abnormalities	Stewart *et al* 1992 (Stewart *et al.*, 1992b) Ware *et al* 1995 (Ware *et al.*, 1995)
TGF-β1	Lethal in embryogenesis	Shull and Doetschman 1994 (Shull *et al.*, 1994)

1.3.3.4 The mouse as a model

In order to evaluate the relevance of different pathways and compounds to an overall system the mouse proves to be a versatile tool to use. In this respect, knockout mice offer exploration to the function of specific genes and their products whereby such an in depth study cannot be carried out on humans. Examples of mouse knockouts that are relevant to reproductive processes are summarised in table 1.3. Although these knockouts give an insight into a physiological situation, these findings in mice cannot be directly applied to the human situation. With regard to the uterus there are many differences between the mouse and the human. The process of decidualisation in humans occurs in anticipation of pregnancy whereas in mice the physical stimulus of implantation is the trigger. This implies that the control of this process in humans is divergent to that in mice. In addition, decidualisation only occurs at the sites of implantation in mice, which is highly specific compared to the dispersed decidual transformation within the human endometrium. Therefore to what extent, for example, IL-11 is essential to decidualisation and implantation in humans may not directly correlate to that occurring in mice (Robb *et al.,* 1998). However, studies using cultures of human ESCs have also indicated IL-11 having a role in decidualisation (Cork *et al.,* 2001; Dimitriadis *et al.,* 2002) therefore illustrating the use of the mouse model as starting place. In the case of the COX-1 knock-out mouse it appears that COX-2 is compensating for the absence of COX-1 and therefore the exact roles of this enzyme become unclear (Reese *et al.*, 1999). This could be occuring in other mouse knock-outs and therefore should be taken into consideration when assessing the results.

With regard to uNK cells the mouse has been used as a model to assess their function in several studies (Ashkar and Croy, 1999; Ashkar *et al.,* 2000; Croy *et al.,* 1991; Croy *et al.,* 2002; Zhang *et al.,* 2003). These studies have focused on knock-out models in particular and the possible functions of uNK cells in humans are discussed in section 1.2.2. However, more recently IL-15 knock-out mice, which are deficient in uNK cells, have demonstrated that these cells are not essential to pregnancy (Barber and Pollard,

2003; Ashkar *et al.,* 2003). What these studies did show was that birth weight was compromised, probably the result of inadequate conversion of the uterine vasculature, (Barber and Pollard, 2003) and this deficiency in weight continued into adulthood (Ashkar *et al.,* 2003). This could have implications on coronary function in the longterm. The work of Barker demonstrated a greater incidence of coronary heart disease and stroke in individuals who had suffered from retarded fetal growth (Barker, 1997a, b and c). In humans the uNK cell may be of greater relevance to the establishment and maintenance of pregnancy than in mice but it may also have implications to intrauterine programming due to its role in blood vessel modification. The distribution of uNK cells is different between mice and humans with them only located at the metrial triangle in mice whereas in humans they are distributed throughout the stroma. These uNK cell knock-out mice do exhibit defective decidualisation (Ashkar *et al.,* 2000) and since this decidual transformation is a pre-requisite to implantation in humans this lack of uNK cells may have a more significant contribution to establishment of pregnancy in this species.

As discussed in Enders, 2000, the mouse proves an economic animal to use as a model, however, differences including the mechanism of implantation and the shape of the lumen exist (Enders, 2000). The mouse is a useful tool and provides a rational starting point for the exploration within the human system or within other animal models more closely related to humans.

1.4 Molecular Contributions to Pregnancy

1.4.1 The Prostaglandin Cascade

Prostaglandin (PG) E_2 and $PGF_{2\alpha}$ are the most abundant PGs in the human endometrium. The major site of production is the glandular epithelial cells both in the endometrium and in early pregnancy (Smith *et al.*, 1988). The production of prostaglandins (PGs) is controlled by two rate-limiting steps, phospholipase A_2 activity

and activity of the two cyclooxygenase enzymes, COX-1 and -2 (figure 1.4). Initially, arachidonic acid (AA) must be liberated from the phospholipid membrane of the cell via PLA_2. This free AA can then be converted to prostaglandin H_2 (PGH_2) by COX-1 and -2. The next stage in the cascade is the action of specific synthases on PGH_2 to direct the production of the five primary prostanoids, namely PGD_2, PGE_2, $PGF_{2\alpha}$ PGI_2 and TXA_2 (Smith *et al.*, 1996; Smith *et al.*, 1996). Three PGE synthases (PGES) have been identified to date, and these include a cytosolic PGES (cPGES) (Tanioka *et al.*, 2000) and two microsomal PGESs, mPGES-1 (Murakami *et al.*, 2000; Mancini *et al.*, 2001) and mPGES-2 (Tanikawa *et al.*, 2002). Aberrant expression of mPGES-1 in conjunction with COX-2 has been postulated to have a role in tumourigenesis (Kamei *et al.*, 2003). In reproductive tissue, PGES has been located in bovine endometrium and its expression correlated with that of COX-2 (Parent *et al.*, 2002). Studies in mice have demonstrated the high expression of mPGES in the stroma that immediately surrounds the blastocyst and have implied a role for this synthase in decidualisation and implantation in rodents (Ni *et al.*, 2002). Conversely, PGs are metabolised and thus deactivated by prostaglandin dehydrogenase (PGDH) enzymes. In the uterus, PGDH has been speculated to have a role in maintaining low concentrations of PGs in the fetal membranes during the majority of pregnancy (Cheung *et al.*, 1992). In some cases of idiopathic preterm labour, a reduction in PGDH expression in the chorionic trophoblast resulting in reduced PG regulation, has been implicated as a causative factor (Sangha *et al.*, 1994). In support of a role for PGs in pre-term labour, inhibition of COX-2 can prevent inflammation-mediated pre-term labour in mice (Gross *et al.*, 2000b). Within the human endometrium, PGDH is located within the cytoplasm of gland cells and levels were found to be highest in the secretory phase but lowest pre-menstrually and during menstruation (Casey *et al.*, 1980). Raised levels in the secretory phase versus the proliferative phase were confirmed in a later study and it was also demonstrated that PGDH activity was significantly increased at 12 months post-insertion of a LNG-IUS and coincided with a rise in PR-A and –B immunostaining (Critchley *et al.*, 1998b). This correlates with the control of endometrial bleeding, implicating both receptor levels and PGs in the process. The role of PGs is supported additionally by a further study that

has demonstrated a decline in PGDH immunostaining in the glands and stroma in women 36-48 hours after receiving the antiprogestin, mifepristone (Hapangama *et al.*, 2002).

PGs have a short half-life and are metabolised rapidly. It is therefore likely that their actions are local to their production site. PGs are classified by their cyclopentane ring and TXA contains an oxane ring. Each group acts via its own distinct receptors (Coleman *et al.*, 1994; Narumiya *et al.*, 1999). These receptors are G protein-coupled receptors with 7 transmembrane domains, located on the plasma membrane. Sequence homology between the different receptors is low, ranging from 20 to 30%, although homology of the specific receptors between species is far higher, 76 – 97% (Narumiya *et al.*, 1999). There are 4 different PGE_2 receptors and these are referred to as EP_{1-4}. The EP_2 and EP_4 receptors act via G_s proteins and have the effect of raising cAMP levels. The EP_3 receptor exists as 7 splice variants (Adam *et al.*, 1994; Sugimoto *et al.*, 2000) with the predominant effect of decreasing cAMP levels. EP_1 acts via a different second messenger, Ca^{2+}, to exert its effects (Kennedy *et al.*, 1982; Watabe *et al.*, 1993).

COX-1 is constitutively expressed in most tissues and in contrast COX-2 is normally absent and only present in a pathological state, for example, COX-2-dependent pathways have been implicated in development of colorectal cancers (Williams *et al.*, 1999) and in eutopic and ectopic pregnancies (Ota *et al.*, 2001). COX-2 can be induced by factors such as lipopolysaccharide (Inoue *et al.*, 1995) and IL-1 (Thomas *et al.*, 2000; Tamura *et al.*, 2002). However, it is becoming apparent that COX-1 can also be induced and it has been shown to be upregulated in cervical carcinomas (Sales *et al.*, 2002). Both of the COX isoforms are inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) and it has been demonstrated in mice and humans that some NSAIDs are preferentially selective for one or other of the COX enzymes (Meade *et al.*, 1993; Gierse *et al.*, 1995). The cause of luteinised unruptured follicle from animal and clinical studies may be related to COX-2 inhibition and a link between NSAIDs and reversible female infertility has been proposed (Stone *et al.*, 2002). In the human endometrium around the time of

implantation, COX-1 and COX-2 are expressed in glandular and luminal cells, and in the luminal and perivascular cells respectively (Marions *et al.*, 1999). When mifepristone was administered, epithelial immunostaining of COX-1 and –2 declined. In addition to illustrating a connection between the PG pathway and steroid hormone action, a function in endometrial receptivity has been suggested (Marions *et al.*, 1999). COX-2 immunoreactivity levels are greater in the premenstrual phase than the mid-secretory phase and this coincides with the recruitment of leukocytes into the endometrium immediately prior to menstruation (Jones *et al.*, 1997).

A variety of PG antagonists have been shown to reduce the implantation site number in mice and it has been suggested that PGs exert effects on both mother and fetus (Biggers *et al.*, 1981). Many studies have assessed the effects of the PG pathway on female fertility and reproductive processes by generating gene knockouts for the various aspects of the cascade. Some of these will be discussed here although it is important to remember that reproductive events such as decidualisation and parturition (Gross *et al.*, 2000a) differ between mice and humans. A double knock-out for COX-1 and COX-2 is lethal, caused by patent ductus arteriosus (Loftin *et al.*, 2001), and therefore it is not possible to study the reproductive consequences in mice of complete COX absence. Targeted disruption of COX-2 in mice leads to problems with the key processes of ovulation, fertilization, implantation and decidualisation (Lim *et al.*, 1997). Studies in mice show two distinct pathways for COX-1 and –2 within the uterus implying independent contributions to uterine PG production but these were also shown to overlap to some extent (Reese *et al.*, 2001). In a previous study on knock-out mice for COX-1 (-/-), on pregnancy day 4 demonstrate reductions in vascular permeability and PG concentration (Reese *et al.*, 1999). However, these reductions were less than those predicted and further investigation revealed a compensatory response by COX-2 demonstrating interaction between the two enzymes. Mice deficient in the EP_2 receptor exhibit a reduced ovulatory number and a reduced fertility rate (Hizaki *et al.*, 1999). No alterations in uterine development are apparent. The lack of EP_4 receptor expression in mice knockouts proves to be fatal soon after birth due to malfunction of the ductus

arteriosus. Mice deficient in cytosolic PLA_2 have reduced fertility, smaller litter sizes and delayed initiation of labour (Bonventre *et al.*, 1997; Uozumi *et al.*, 1997).

1.4.1.1 PGE_2 as an Immune Modulator

PGs have been implicated as modulators of immunity (Harris *et al.*, 2002) and in support of this PGE_2 can inhibit many immune cells such as T cells (Goodwin *et al.*, 1983) and neutrophils (Fantone *et al.*, 1983). Within human semen high concentrations of PGE_2 are contained and this is believed to aid reproductive success via inhibition of female immune responses (Kelly, 1991). Decidual stromal cells are a source of PGE_2 and this PG has been shown to have an inhibitory effect on NK cells via the IL-2 and IL-15 receptors (Parhar *et al.*, 1989; Joshi *et al.*, 2001). Addition of indomethacin or anti-PGE_2 antibody to NK cells *in situ* revealed an up-regulation of the IL-2 receptor α chain, IL-2 production and an increased anti-trophoblast killer activity (Parhar *et al.*, 1989). PGE_2 also has the ability to alter the cytokine profile of cells and favour a Th-2 response in preference to a Th-1 reaction by stimulating IL-10 and inhibiting IL-12 production in blood monocytes (van der Pouw Kraan *et al.*, 1995).

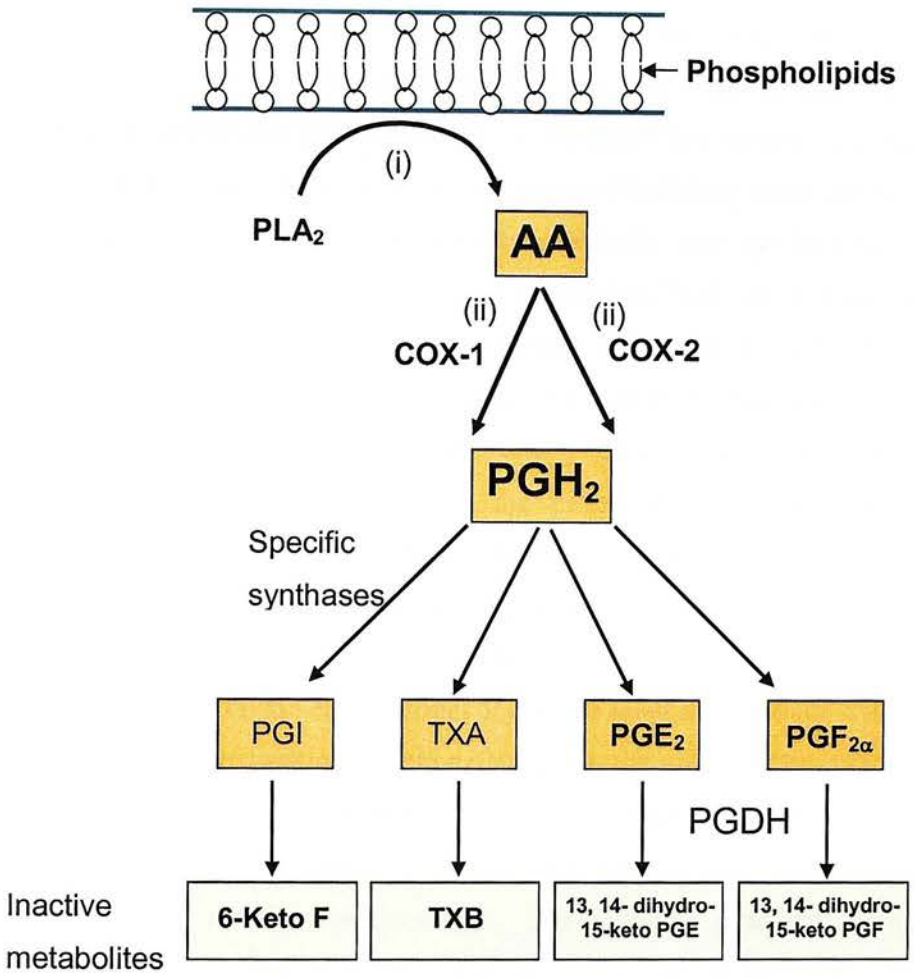


Figure 1.4

The prostaglandin cascade illustrating the two rate-limiting stages (i) PLA_2 activity to liberate free arachidonic acid (AA) from membrane phospholipids and (ii) COX-1/-2 activity in the conversion of AA to PGH_2. The PGs and TXAs then act on their specific GPCRs.

1.4.2 Cyclic Adenosine Monophosphate (cAMP)

Intracellular second messengers allow the transmission of signals to pass within a cell and produce an amplification of the signal. One of these important messengers is cAMP. It is produced from ATP when adenylyl cyclase is activated. The original signal is then amplified via a stimulated protein kinase cascade. Phosphodiesterases act to breakdown cAMP and thus counteract the effects of this second messenger. Levels of cAMP can be raised by the presence of phosphodiesterase inhibitors such as rolipram. Activators of adenylyl cyclase including forskolin also produce the end result of increasing cAMP levels. Within the endometrium PGE_2 is an activator of adenylyl cyclase and relaxin inhibits phosphodiesterases (Bartsch *et al.*, 2001) thus raising cAMP levels. The connection between cAMP and the ovarian sex hormones could be partly via the progesterone receptor. In the breast cancer cell line, T47D, CAT assays have shown the ability of cAMP to potentiate the progesterone-dependent activation of the progesterone receptor but only in the presence of progesterone (Edwards *et al.*, 1993). In post-menopausal women the antiprogestin, RU486, can act as an agonist in the endometrium but only when in the presence of progesterone (Gravanis *et al.*, 1985). The effects of RU486 on the endometrium of castrate monkeys has been studied, and RU486 was found to have weak progestational effects in the proliferative phase but strong anti-progestational effects in the secretory phase (Koering *et al.*, 1986). Experiments on T47D cells also demonstrated that when RU486 was added in combination with cAMP a partial agonist activity was produced. This implies cross-talk between a second messenger signal transduction pathway and a nuclear receptor but whether this also occurs within the endometrium has yet to be established.

Within the human endometrium, cAMP is the main second messenger involved in the PGE_2 signal transduction pathway (Frank *et al.*, 1994). Evidence for the involvement of cAMP and its analogues in the decidualisation of human endometrium has been proposed (Tang *et al.*, 1993a; Brosens *et al.*, 1996; Brar *et al.*, 1997; Brosens *et al.*, 1999). Experiments using cultures of ESCs have shown that prolactin expression is

dependent on activation of the PKA pathway (Brar *et al.*, 1997; Telgmann *et al.*, 1997). Direct use of cAMP results in an increase in prolactin mRNA expression and protein release (Tang *et al.*, 1993a; Brosens *et al.*, 1996; Brar *et al.*, 1997; Brosens *et al.*, 1999) and this elevation is a permanent response (Telgmann *et al.*, 1998). They demonstrated the ability of cAMP to synergise with a synthetic progestin to result in stromal differentiation and an increase in prolactin levels. Progesterone treatment alone proved to be a weak inducer of the decidualised phenotype. High intracellular cAMP levels appear essential by sensitising the cells to progestins and thus providing maximal prolactin expression. Deletion of the region between position –332 and –270 in the dPrl promoter caused a strong reduction in the ability of cAMP to induce prolactin expression implying the importance of this particular region in its activation (Telgmann *et al.*, 1998).

1.4.3 Interleukin-15 (IL-15)

IL-15 was initially discovered as a stimulator of T-cell proliferation (Grabstein *et al.*, 1994). It has a molecular weight of 14-15 kDa and is a member of the 4 α-helix cytokine family. The receptor complex for IL-15 is composed of three subunits. The α-chain is unique to its receptor but both the β- and γ-chains are also common to the IL-2 receptor (Carson *et al.*, 1994; Grabstein *et al.*, 1994). Two forms of IL-15 exist due to alternative splicing resulting in a long signal peptide (LSP), 48-AA in length, and a short signal peptide (SSP), 21-AA long (Fehniger *et al.*, 2001) (figure 1.5). The SSP IL-15 is restricted to the nuclear and cytoplasmic compartments of the cell whereas the LSP has been detected in the golgi apparatus and is thought to follow a secretory route (Tagaya *et al.*, 1997).

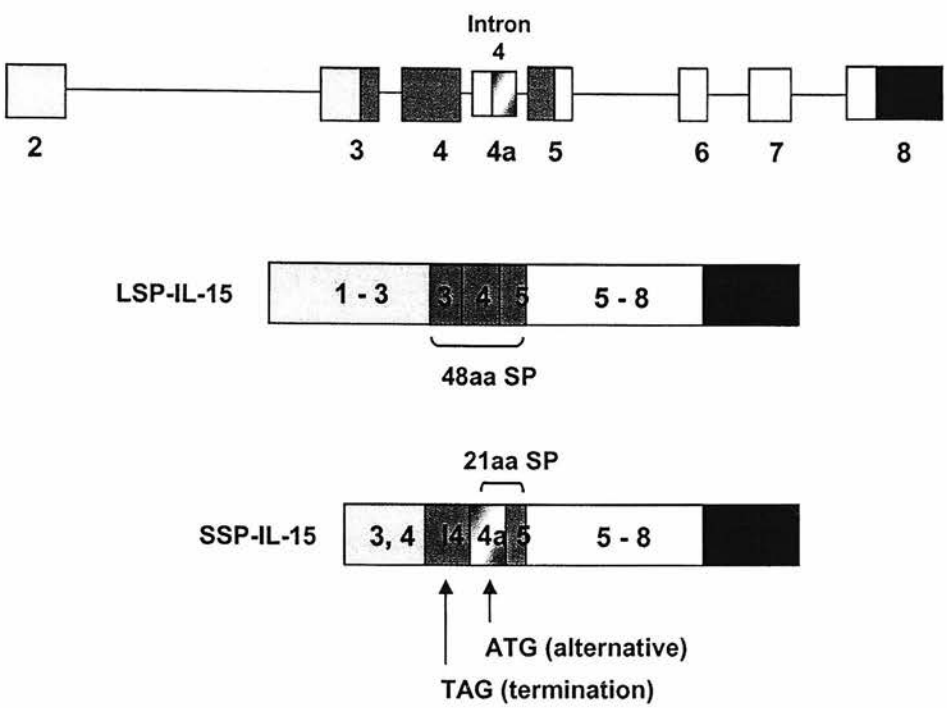


Figure 1.5

The generation of the two isoforms of IL-15, SSP and LSP, from the human IL-15 gene locus. Diagram recreated from Waldmann *et al* 2001 (Waldmann *et al.*, 2001).

1.4.3.1 Systemic IL-15

Human IL-15 has 97% sequence homology compared with simian IL-15 and 73% homology with murine IL-15 (Anderson *et al.*, 1995). This conservation in IL-15 between species suggests an important biological role. This is compounded by its wide tissue distribution within humans including skeletal muscle, heart, kidney, placenta and uterus (Grabstein *et al.*, 1994). Inappropriate production of IL-15 can lead to the manifestation of a number of human diseases. An implied involvement of IL-15 in autoimmune and inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease support an immune connection with IL-15 (Fehniger *et al.*, 2001). In contrast, the functions of IL-15 stretch beyond the immune system. In skeletal muscle it appears to have anabolic properties associated with skeletal muscle fiber hypertrophy (Quinn *et al.*, 1995). However, knockout mice studies have shown no defects in muscle development implying a non-critical role in this tissue (Kennedy *et al.*, 2000). Detection of IL-15 has been reported in human cortical tubular epithelial cells. Both the message and protein levels are increased by the Th-1 cytokine IFN-γ and by ligation with CD40 and it has been suggested that this knowledge may be important in kidney transplantation (Weiler *et al.*, 1998; Weiler *et al.*, 2001).

1.4.3.2 The Reproductive Relevance of IL-15

Although the actions of IL-2 and IL-15 are reported to be similar, their tissue distribution is distinct. The uterus and placenta were found to be negative for IL-2 (Jokhi *et al.*, 1994b; King *et al.*, 1995) and so further investigation into other cytokines that could stimulate uNK cells was undertaken. The novel cytokine, IL-15 was discovered via detection of both the mRNA (Okada *et al.*, 2000b; Verma *et al.*, 2000) and protein (Kitaya *et al.*, 2000). Gene profiling has demonstrated an upregulation of IL-15 during the human "implantation window" compared with the late proliferative phase. The IL-15 precursor, IL-15 and the α chain of the IL-15 receptor are all increased by at least three-fold (Kao *et al.*, 2002) and this supports the role of IL-15 in

the process of implantation. Other studies have also confirmed higher levels of IL-15 mRNA expression in the secretory phase versus the proliferative phase with highest expression levels in the mid-late secretory phase (Kitaya *et al.*, 2000; Okada *et al.*, 2000b; Verma *et al.*, 2000; Chaouat *et al.*, 2002). Immunohistochemistry localised IL-15 to the glandular and luminal epithelium during the proliferative phase and in the mid-late secretory phase to the perivascular stromal cells (Kitaya *et al.*, 2000). Studies on stromal cells grown *in vitro* imply that production of this cytokine is partly under hormonal control (Okada *et al.*, 2000a). However, the presence of IL-15 is not limited to the menstrual cycle but is also abundant in first trimester decidua (Kitaya *et al.*, 2000; Okada *et al.*, 2000b).

The human placenta is an important source of IL-15. Studies utilising a trophoblast invasion model, JEG-3, discovered IL-15 has the ability to enhance trophoblast invasion and migration *in vitro* (Zygmunt *et al.*, 1998) and it is postulated to be involved in this process *in vivo*. It is apparent that the ratio of IL-13: IL-15 is important in controlling this process *in vivo* since it has been discovered that women experiencing recurrent spontaneous abortion (RSA) expressed raised levels of IL-13 and IL-15 in endometrial stromal and epithelial cells with the ratio being in favour of IL-13 (Chegini *et al.*, 2002). In general terms it is accepted that a Th-2 response is beneficial to pregnancy (Wegmann *et al.*, 1993), however, the rise in IL-13 expression over that of IL-15 in RSA endometrium implicates complexity to this classification (Chegini *et al.*, 2002). The general hypothesis that Th-1 cytokines are detrimental and Th-2 cytokines are beneficial to pregnancy is probably an over-simplification (Chaouat *et al.*, 2002). This is of particular importance given the role of IFN-γ in the establishment of normal pregnancy in the mouse (Ashkar *et al.*, 1999; Ashkar *et al.*, 2000).

1.4.3.3 Studies on IL-15 Null Mice

Knockout mice for IL-15 (-/-) have no NK cells present and this suggests a role for IL-15 in the development and maturation of these cells (Kennedy *et al.*, 2000). Studies

specifically within the uterus have shown with knock-out studies in mice that mutant mice lacking uNK cells display irregular oestrous, deficient decidual formation and thickened blood vessel walls (Miyazaki *et al.*, 2002). In addition to these defects, a reduced placental size occurs in uNK cell deficient mice (Greenwood *et al.*, 2000). This supports the theory that these cells are involved in transformation of uterine vasculature (King *et al.*, 1990). In humans, inadequate artery transformation can result in pre-eclampsia, intra-uterine growth retardation (IUGR) and still birth and with the case of IUGR this programming *in utero* can be associated with cardiovascular diseases in adult life (Barker, 1997a; Barker, 1997b).

1.5 Hypothesis and Aims

Previous studies have identified various factors that are involved in the decidualisation of endometrial stroma, a process that is essential to a successful pregnancy in humans. The accumulation of uNK cells across the secretory phase coincides with this decidual transformation of the stromal compartment. The regulation of uNK cells and their specific functions in the human uterus are not fully understood although it is likely they are closely linked to the ESC that is under progesterone control. From the results of previous research discussed in section 1.2.2.1, a hypothesis of these speculative actions has been summarised in figure 1.6.

Paracrine interactions between uNK cells and ESCs via cytokines are likely to be essential to the respective development and functions of both cell types. Specific products of uNK cells, including IFN-γ and IL-10, may be modulating the function of ESCs in addition to maintenance of inflammatory and immune homeostasis. At this point in time the ESC cell will be transforming via decidualisation and products of the uNK cells may be modulating this conversion reaction. Reciprocally, cytokine production by ESCs will provide communication with uNKs and possibly amend their cytokine production. Feedback between these cell types will be essential to fine-tuning the control of these actions and thus establish an environment correctly prepared for pregnancy.

Prostaglandins are considered important to many of the key reproductive events in the human uterus although their exact effects are not yet clearly defined. Their actions are controlled at multiple levels within cells by the activity of various enzymes and this represents a complex cascade of events. PGE_2 is involved in decidualisation of the endometrial stroma, a process in humans essential to successful implantation. Expansion in our knowledge of the changes occurring to the PGE_2 pathway will provide a greater understanding of the mechanisms controlling the decidual transformation. This process is likely to be tightly regulated with modifications at a number of control points

along the cascade. The transport of PGE_2 across the cell membrane is a further control mechanism post-translationally and will be relevant to transport out of and into cells. In addition to actions on ESCs, PGE_2 can act to modify the actions of IL-15 on the cell surface of NK cells at the receptor level. PGE_2 may also be regulating the production of cytokines by uNK cells via actions on the IL-15 receptor or directly on its own receptors at the surface of the cell.

The Main Aims of this Research Project:

1. Further investigate decidualisation of human ESCs *in vitro* and explore the functional link between IL-15 and decidualisation.

2. Assess the changes in the prostaglandin cascade during decidualisation of ESCs.

3. Assess the potential factors that are involved in the regulation of IL-15 mRNA expression and protein secretion in ESCs *in vitro* and in the human endometrium and decidua.

4. Investigate the effects of PGE_2 and IL-15 on uNK cells *in vitro*.

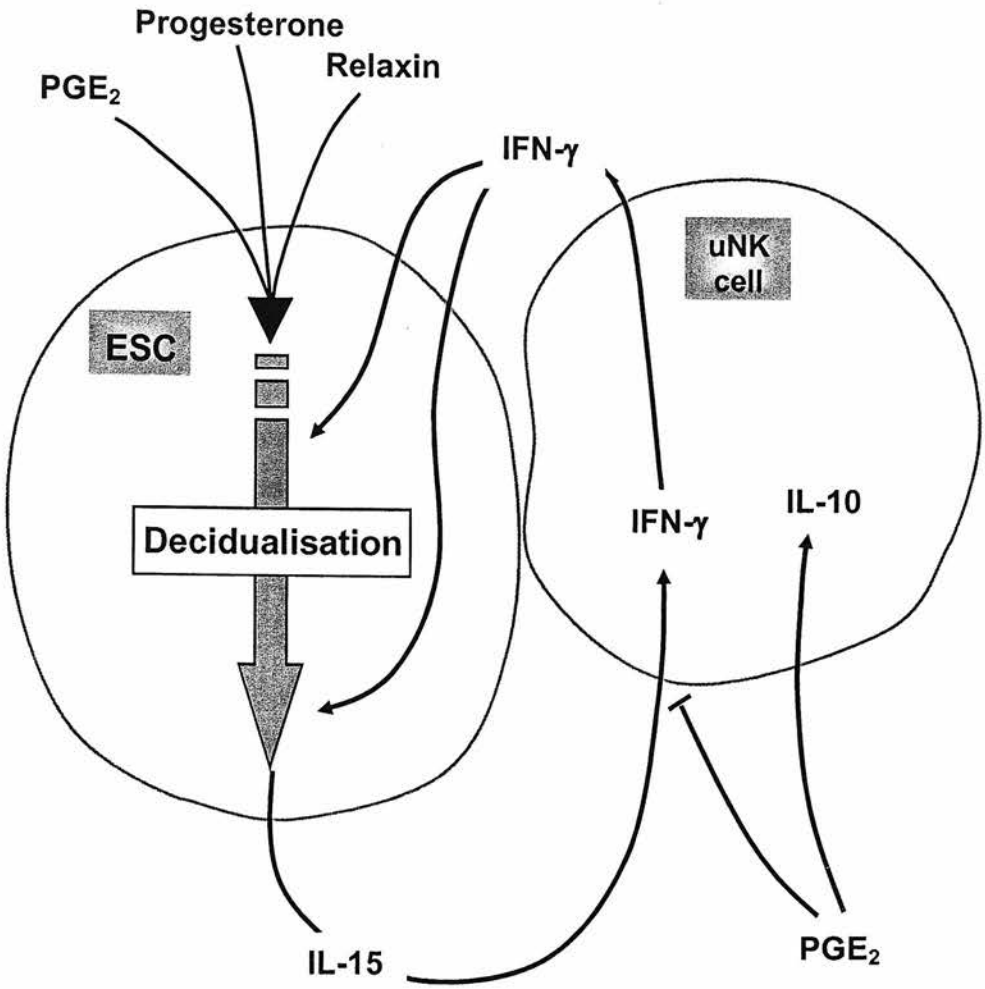


Figure 1.6

The hypothesised interactions between uNK cells and ESCs in the human endometrium/decidua during the secretory phase and in early pregnancy implicating the indirect action of progesterone on uNK cell function via the ESC. Studies on blood NK cells demonstrated that it is the $CD56^{bright}$ NK cells that may be the major producers of cytokines (Cooper *et al.*, 2001a). uNK cells may therefore represent an important uterine cell able to be stimulated to produce a variety of inter-cellular mediators.

2. General Methods

2.1 Collection, Processing of Human Uterine Tissue and Ethics

Human endometrial specimens were collected from women of reproductive age (n = 77) who were undergoing gynaecological procedures for benign indications. All women had regular menstrual cycles with a length of between 25-35 days and had not received exogenous hormones or used an intrauterine contraceptive device in the 3 months prior to surgery. For all endometrial biopsies analysed, the stage of the menstrual cycle was consistent with the patient's reported last menstrual period and histological dating using the criteria of Noyes *et al* (Noyes *et al.*, 1950). Samples were classified as menstrual (M), proliferative (P), and early (ES), mid (MS) and late secretory (LS) phases for the purposes of Taqman Q RT-PCR. Any cases with severe uterine pathology, for example, polyps or large fibroids, were excluded. All subjects had a serum sample collected at the time of surgery for the determination of circulating oestradiol and progesterone levels by RIA. All samples were consistent with the designated cycle stage based on morphological criteria and last menstrual period (see table 2.1) (Critchley *et al.*, 2002).

Decidual tissue was collected by two different methods for varying experimental procedures. For the purpose of the analysis of mRNA expression across the cycle decidual (n = 5) and trophoblast (n = 4) specimens were obtained from women who had undergone surgical termination by vacuum aspiration, during the first trimester of pregnancy. All patients had the termination performed under general anaesthesia. Prior to vacuum aspiration, decidual tissue distant from the implantation site was removed by gentle curettage of the uterine lining. Presence and absence of decidual parietalis and trophoblast respectively was confirmed by examination of haematoxylin and eosin stained tissue sections. Tissue sections were also stained with cytokeratin to confirm the presence or otherwise of trophoblast cells within the decidual tissue (Critchley *et al.*, 1996). The endometrial tissue was collected in sterile Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, Poole, Dorset, UK). The decidual tissue samples were processed for messenger RNA (mRNA) extraction alone. The endometrial samples were divided and processed as follows: (i) homogenisation in Tri Reagent® (a

monophase solution containing phenol and guanidine thiocynate) to allow mRNA isolation from DNA and protein; (ii) fixation in 10% neutral buffered formalin (NBF) overnight at 4°C followed by storage in 70% ethanol preceding wax embedding; and (iii) separation of endometrial stromal cells (ESCs) by sedimentation (see section 2.2). A second method of decidua collection was utilised in order to collect tissue for the uterine Natural Killer cell separation procedure. Insufficient amounts of tissue could be collected via the former method. In order to collect larger volumes of decidual tissue the total tissue yield from the suction curettage procedure (used to evacuate uterine contents) were collected. Decidual tissue (n = 15) was selected by macroscopic inspection from the products of the termination aspiration procedure (this process was performed by our trained, Research Sister). Thereafter decidual tissue was collected in sterile RPMI 1640 medium (Sigma, Poole, Dorset, UK) in preparation for the uterine Natural Killer cell separation procedure. A small section of tissue was placed in RNA Later (Ambion Europe Ltd., Cambridgeshire, UK) in order that mRNA could be later extracted. A second small section of tissue was placed into 10% NBF and following fixation for 24 hours samples were placed in 70% ethanol preceding wax embedding. Decidua was only collected from women with a pregnancy of gestation age between 7 and 10 weeks.

Informed patient consent was obtained prior to all tissue collection by a dedicated Research Nurse. Ethical local research approval had been previously granted by the Lothian Research Ethics Committee for all studies.

Cycle days/ weeks of gestation (decidua)	Cycle Stage	Number of tissue samples	Oestradiol (pmol/l) Median (range)	Progesterone (nmol/l) Median (range)
1 - 4	Menstrual	9	136 (57 – 306)	3.4 (1.6 – 4.4)
5 - 7	Early-proliferative	1	669	1.5
8 - 10	Mid-proliferative	19	570 (193 – 1235)	1.8 (1.3 – 15.5)
11 – 13	Late-proliferative	7	599 (211 – 1010)	4.4 (2.6 – 10.1)
14 – 18	Ovulatory/ Early-secretory	16	425 (264 – 1323)	15.4 (1.5 – 63.3)
19 – 23	Mid-secretory	9	368 (120 - 650)	33.7 (8.4 – 71.3)
24 – 28	Late-secretory	7	398 (189 – 477)	8.9 (1.1 – 28.1)
7 - 10	First trimester decidua	20	NA	NA

Table 2.1

Details of the endometrial and decidual biopsies studied in this research project. The median and range of serum oestradiol and progesterone concentrations are included.

2.2 Endometrial Cell Culture

Endometrial specimens (n = 28) were separated into epithelial and stromal cell populations by a sedimentation procedure. Specimens were initially washed in phosphate buffered saline and sliced into 1-2mm pieces using scalpel blades. These were re-suspended in 2ml PBS and digested in collagenase (Sigma) for 80 minutes at 37°C. An 18 gauge needle was used to facilitate tissue breakdown. The cells were then pelleted by centrifugation and re-suspended in 12ml RPMI 1640 and left for 5 minutes to settle. The top 10ml were removed as the population of endometrial stromal cells (ESCs). A Fluorescence Activated Cell Sorter (FACS) had been used to verify the purity of cells separated by this technique (see section 2.3). The ESCs were further cultured in RPMI 1640 medium (Sigma) supplemented with 10% foetal calf serum (FCS) (Mycoplex; PAA, Teddington, UK), penicillin (50 μg/ml; Sigma), streptomycin (50 μg/ml; Sigma) and gentamycin (5 μg/ml; Sigma). The endometrial specimens used in the cell culture experiments were predominantly from the proliferative phase. However, due to a shortage of specimens it was necessary to use some from the early secretory phases also. These specimens will have been exposed to progesterone *in vivo* although those from the proliferative phase had not. It was therefore necessary to grow all of the cultures in the prescence of progesterone prior to being used in the experiments so that all cultures had been exposed to progesterone. Cells were cultured in $75cm^3$ culture flasks (Corning Incorporated, Corning, NY) for a minimum period of 5 days and allowed to reach confluence in the presence of oestradiol (10^{-7} M), Medroxyprogesterone acetate (MPA) (10^{-6} M) and basic fibroblast growth factor (bFGF) (5ng/ml). These supplements also provide optimum conditions for ESC growth (Irwin *et al.*, 1991).

2.3 Fibroblast Cell Purity

Cultures of ESCs were grown for two weeks before the purity of fibroblasts in the cultures (n = 6) was determined by a Fluorescence Activated Cell Sorter (FACS)

(Beckmann Coulter). This was achieved by using a human fibroblast antigen Ab-1 (Oncogene). About 1 million ESCs were taken from an ESC culture following trypsinisation, re-suspended in 200µl Cell Separation Media (CSM) (PBS 0.5% bovine serum albumin; 2mM EDTA) and split between three 1.5ml Eppendorf tubes. Into one tube 2µl of the fibroblast antibody was added and mixed and the tubes were left on ice for 30 minutes. The tubes were spun at 4000rpm for 1 minute to pellet the cells, which were re-suspended in 1ml CSM. This wash was repeated twice more. To two tubes 100µl CSM and 10µl anti-mouse IgG whole molecule with FITC conjugated (Sigma) was added and cells re-suspended. The remaining tube is used as a "blank". The tube exposed to only the anti-mouse IgG whole molecule with FITC conjugated is the negative isotype-matched control. The tubes were wrapped in foil to prevent light exposure and kept on ice for 30 minutes. Tubes were washed as before and re-suspended in 500µl FACs buffer (PBS 0.1% Azide; 1% FCS). Before passing the cells through the FACS machine the cell suspensions were passed though cell strainers (Becton Dickinson Labware Europe, France) to ensure a single cell suspension. See figure 2.1 for an example of the FACS reports.

2.3.1 Fluorescence Activated Cell Sorter (FACS)

A Coulter ® EPICS® XL™ Flow Cytometer was the FACs machine used to measure the purity of the cells. This machine allows the purity of a specific cell type within a mixed population of cells to be measured. The cells are passed through an aperture as single cells. The laser beam detects cells and registers them as an event. The laser beam is able to determine two properties of each cell: the granularity and cell size. The granularity is read by the amount of side scatter of the laser beam and the cell size determined by the degree of forward scatter. The third measurement made by the laser beam is fluorescence, in this case fluorescein. The three measurements combined illustrate the distinct populations of cells that are present and the percentage of fluorescent gives the percentage of fibroblast-positive cells in the sample.

Figure 2.1

A representative example of the FACS recordings for the fibroblast purity of ESCs after 2 weeks in culture. The isotype-matched negative control (**A**) and the sample incubated with the fibroblast antibody (**B**). On graph (i) the y axis is the log of the forward scatter and the x axis is the log of the side scatter. The total number of counts is illustrated on this graph and the region within the red line represents the gated area of cells analysed. The remainding points that are outside of this region are likely to be cell debris or red blood cells and are therefore excluded from analysis. On graph (ii) the y axis is the total cell count and the x axis is the FITC value – the total level of fluorecence detected on the cells counted within the gated region in graph (i). The region represented by the green line indicates the number of cells that are positive for FITC. This is minimal in figure A (ii) (the negative control) whereas in figure B (ii) the majority of the cells are positive for FITC.

A **MRC EDINBURGH**

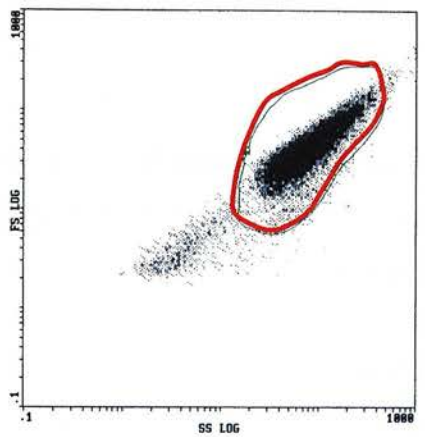
COULTER(R) EPICS(R) Acquisition Flow Cytometry Report

14May02 11:04:03
fibro 15.3.02
Z0012760
Endo150 neg 14.5.02 fibrc
31 seconds. 10817 events
Stop Count: 10000 events, histogram 2

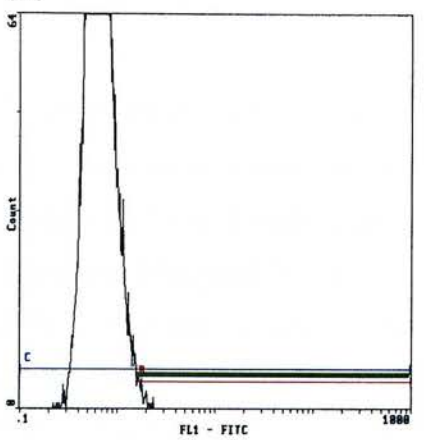
OP ID: SJD

Initial cytosett. from prot. fibro 15.3.02

(i)



(ii)



Stats: Normalized, Listgating: Disabled

Color equations

Hist	Region	ID	%	Count	Mn X	Mn Y	PkPosX	PkPosY	PkCnt	FPCVX	FPCVY
1	A	A	92.4	10000	77.2	38.5	71.5	40.2	65	61.62	56.00

Hist	Region	ID	%	Count	Mn X	Md X	PkPosX	PkCnt	HPCV	Min	Max
2	B	B	1.34	134	2.12	1.80	1.62	8	0.69	1.61	1024
	C	C	100	10000	0.673	0.648	0.602	150	18.25	0.102	996.7

B **MRC EDINBURGH**

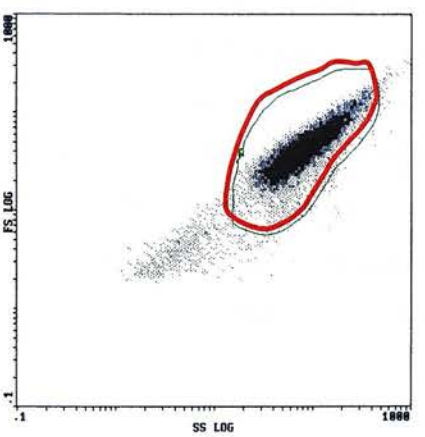
COULTER(R) EPICS(R) Acquisition Flow Cytometry Report

14May02 11:05:38
fibro 15.3.02
Z0012761
Endo 150 pos 14.5.02 fibrc
42 seconds. 10668 events
Stop Count: 10000 events, histogram 2

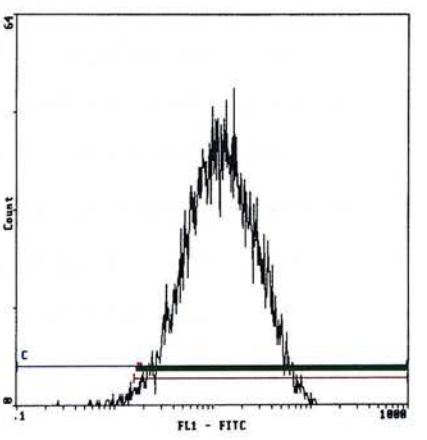
OP ID: SJD

Initial cytosett. from prot. fibro 15.3.02

(i)



(ii)



Stats: Normalized, Listgating: Disabled

Color equations

Hist	Region	ID	%	Count	Mn X	Mn Y	PkPosX	PkPosY	PkCnt	FPCVX	FPCVY
1	A	A	93.7	10000	80.7	39.7	71.5	40.2	62	63.05	55.95

Hist	Region	ID	%	Count	Mn X	Md X	PkPosX	PkCnt	HPCV	Min	Max
2	B	B	98.9	9886	12.5	12.5	10.9	57	6.44	1.61	1024
	C	C	100	9999	12.2	12.4	10.9	57	6.44	0.102	996.7

2.4 Messenger RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

2.4.1 Messenger RNA Extraction

The medium on the treated cell cultures was pipetted off and either frozen at –20°C or maintained at 4°C if being used immediately. Cells were washed in PBS twice to remove the remnants of medium. Onto each well of a 12-well plate, 0.5ml tri-reagent was added whereas if 6-well plates or small flasks had been used, 1ml of tri-reagent per well or flask was added. Cells were left for 5 minutes to allow rupture of cell membranes to take place. The contents of the wells/flasks were then transferred to phase lock gel tubes (Eppendorf, Hamburg, Germany) and kept on ice for the remaining extraction. Chloroform was added at 200µl/ml and the tubes shaken vigorously for several seconds. They were spun in a pre-cooled, refrigerated centrifuge at 4°C, 14000rpm for 20 minutes to remove the organic phase containing protein and DNA that become trapped beneath the gel layer. The top aqueous phase containing the mRNA was poured into labelled 1.5ml eppendorfs. Into each tube 500µl of isopropanol was added and tubes were inverted a couple of times before being left on ice for 1 hour. They were spun in a pre-cooled, refrigerated centrifuge at 4°C, 14000rpm for 15 minutes to pellet the mRNA. The majority of the isopropanol was removed using a sterile Pasteur pipette and 500µl of 70% ethanol added to each Eppendorf. After being inverted the tubes were spun for 5 minutes in a pre-cooled, refrigerated centrifuge at 4°C, 14000rpm. The ethanol was removed from each tube and between 20-50µl RNA Storage Buffer (RSB) was added depending on the size of the mRNA pellet. Samples were left to dissolve in the RSB for a minimum of 30 minutes.

The concentration of total RNA in the samples was then measured using a Biotech photometer (WPA, Cambridge, UK). Each sample was vortexed and 2.5µl removed, which in turn was diluted in 2ml distilled water. All tubes were thoroughly mixed before measuring the wavelength at both 260nM and 280nM on the photometer. A

blank used to zero the machine consisted of 2.5µl RSB diluted in 2ml of distilled water. All samples were then diluted in Diethylpyrocarbonate-treated water (Depc water) to a concentration of 100ng/µl.

2.4.2 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Messenger RNA samples (at 100ng/µl) were reversed transcribed using a multiscribe reverse transcriptase (1.25 IU/µl), random hexamers (2.5 µmol/l), $MgCl_2$ (5.5 mmol/l), dNTPs (1mmol/l) and an RNAase inhibitor (0.4 IU/µl) was added (PE Biosystems, Warrington, UK). All reactions consisted of 16µl of reverse transcription mix per tube and 4µl of mRNA. Once the contents of the tube were mixed, 50µl of mineral oil was added to prevent evaporation of the reaction mix during the reverse transcription process. Samples were incubated for 20 minutes at 25°C, 60 minutes at 42°C and then at 95°C for 5 minutes (Omnigene PCR machine, UK). The resulting cDNA was then diluted 2.5x with TE buffer (10mmol/l Tris pH8.0 and 1mmol/l EDTA in DEPC water) and were stored at 4°C.

The precision of the reverse transcription (RT) reaction was calculated by a colleague, Elena Faccenda (laboratory research support). An mRNA sample was taken and 8 RT reactions set up in 8 separate tubes. These were compared in a single PCR run on one primer and probe set. The precision was found to be 3.65%.

2.5 Quantitative Real-time-PCR (Q RT-PCR)

This procedure allows the quantitative detection and measurement of a specific sequence of complementary DNA (cDNA). Primers and probes were designed individually for each DNA target sequence using the Primer Express computer program. The probe has a reporter dye and quencher dye attached to the 5' and 3' ends respectively. Whilst intact, the close proximity of the reporter to the quencher suppresses fluorescence of the reporter. If the target sequence is present the probe anneals to the cDNA in between the

forward and reverse primer sites and this is referred to as the polymerisation step. The sequence of either strand must be complementary to the probe for it to attach. The Taq polymerase has nuclease activity in the 5' to 3' direction and cleaves the probe in between the reporter and quencher. This can only take place if the probe has hybridised to the target sequence of cDNA. Cleavage of the probe allows separation of the reporter dye from the quencher dye with the result being an increase in fluorescence. This increase in fluorescence allows the accumulation of PCR products to be measured directly.

The FAM Ct is the cycle number when the fluorescent signal crosses an arbitrary threshold value. The 18-S is a measure of the mRNA content of the sample and is used as an internal control for mRNA variation between samples. The ΔCt is the difference between the FAM Ct and the 18-S reading and enables the amplified signal to be normalised against the total mRNA content. The mean ΔCt between the duplicates on the PCR plate was calculated. This was then used to calculate the ΔΔCt which is the difference between the ΔCt of a treated sample relative to the control in that experiment. Within each experiment the ΔCt was related to its own control. The $^{2^-}$ΔΔCt is then calculated which shows the fold increase or decrease in mRNA expression of the samples in relation to their control with each control always having a $^{2^-}$ΔΔCt of 1.

All primer and probes sets used on the ABI Prism 7700 PCR machine were purchased from Biosource (Belgium). The sequences for these are listed in table 2.2. Primers were diluted to 250μM and probes to 50μM in TE buffer (10mM Tris; 1mM EDTA in Depc H_2O). A PCR master mix was made up using the Stratagene Brilliant Quantitative PCR Core Reagent kit (Amsterdam, Netherlands) (7.2mM $MgCl_2$; 1.6mM Stratagene dNTP mix; 1.6mM Boehringer dNTP mix; 0.05U/μl Taq Polymerase; 2x PCR Buffer and 0.06% Reference dye diluted in Depc H_2O). Sample to be tested were ran in duplicate wells on the PCR plate (Applied Biosystems, UK). For each sample, a tube containing 45μl PCR Master Mix and 5μl cDNA was mixed and 23μl pipetted into two separate

wells on the PCR plate. Two wells containing 5µl of Depc H_2O in place of cDNA were added to each run to serve as a negative control.

The primer and probe sets used were validated with respect to the linearity of the response. Graphs of the log of total RNA in ng were plotted against the mean ΔCt value of three replicates. The regression line of the graph (y) needed to be < 0.1 in order that the primers and probe could be validated (Figure 2.2). The intra-assay variation was calculated as a precision value (relative standard deviation) calculated from the mean of six replicates of identical cDNA ran on the PCR machine in the same run. This value is expressed as a percentage. (See table 2.3).

Table 2.2

Details of the primer and probe sets used in Q RT-PCR in this study. The accession number and sequences for the primers and probe are detailed.

Amplicon	Accession Number	Forward Primer	Reverse Primer	Probe
β-actin	X00351	TCACCCACA CTGTGCCCA TCTACGA	CAGCGGAAC CGCTCATTG CCAATGG	ATGCCCCCCC CATGCCATCC TGCGT
CD40L	Z15017	GTGCTTCGG TGTTTGTCA ATGT	CAGCCTGCA AGGTGACA CTGT	ACTGATCCA AGCCAAGTG AGCCATGG
COX-1	S78220	TGTTCGGTG TCCAGTTCC AATA	ACCTTGAAG GAGTCAGGC ATGAG	CGCAACCGC ATTGCCATG GAGT
COX-2	M90100	GTGTTGACA TCCAGATCA CATTTGA	GAGAAGGCT TCCCAGCTTT TGTA	TGACAGTCCA CCAACTTACA ATGCTGACTA TGG
EP2 Receptor	U19487	GACCGCTTA CCTGCAGCT GTAC	TGAAGTTGC AGGCGAGCA	CCACCCTGC TGCTGCTTC TCATTGTCT
EP4 Receptor	D28472	ACGCCGCCT ACTCCTACA TG	AGAGGACGG TGGCGAGAA T	ACGCGGGCT TCAGCTCCT TCCT
IFN-γ	X13274	CCAACGCA AAGCAATA CATGA	TTTTCGCTT CCCTGTTTT AGCT	CTCATCCAA GTGATGGCT GAACTGTCGC
IGFBP-1	M59316	CACAGGAG ACATCAGG AGAAGAAA	ACACTGTCT GCTGTGATA AAATCCAT	TTCCAAATTT TACCTGCCAA ACTGCAACAA

Amplicon	Accession Number	Forward Primer	Reverse Primer	Probe
IL-15	U14407	GTATTGTA GGAGGCAT CGTGGAT	TAGCACTG GATGGAAA TACTTCTCA	ATGGCTGC TGGAAACC CCTTGCC
PDE3a	M91667	GCAGCTGT GGCAGACC ATATT	CCGAGTGG CTACCCCA CTT	AATCCTGC TGATGAGC CCCTGGAG A
PDE4b	L20971	CCTTCAGTA GCACCGGA ATCA	CAAACAAA CACACAGG CATGTAGTT	AGCCTGCA GCCGCTCC AGCC
PGTP	U70867	CTGTGGAG ACAATGGA ATCGAGTA	CAAATAGA TCAGTTGC TTGGAGGTT	CTCCCCTT GCCATGC CGGC
Progesterone Receptor (genomic)	M15716	CAGTGGGC GTTCCAAA TGA	TGGTGGAAT CAACTGTAT GTCTTGA	AGCCAAGC CCTAAGCC AGAGATTC ACTTT
Prolactin	NM 000948	GCCCCGGA GGCTATCC TA	TCAGCTCCA TGCCCTCTA GAA	CCAAAGCT GTAGAGAT TCAGGAGC AAACCA

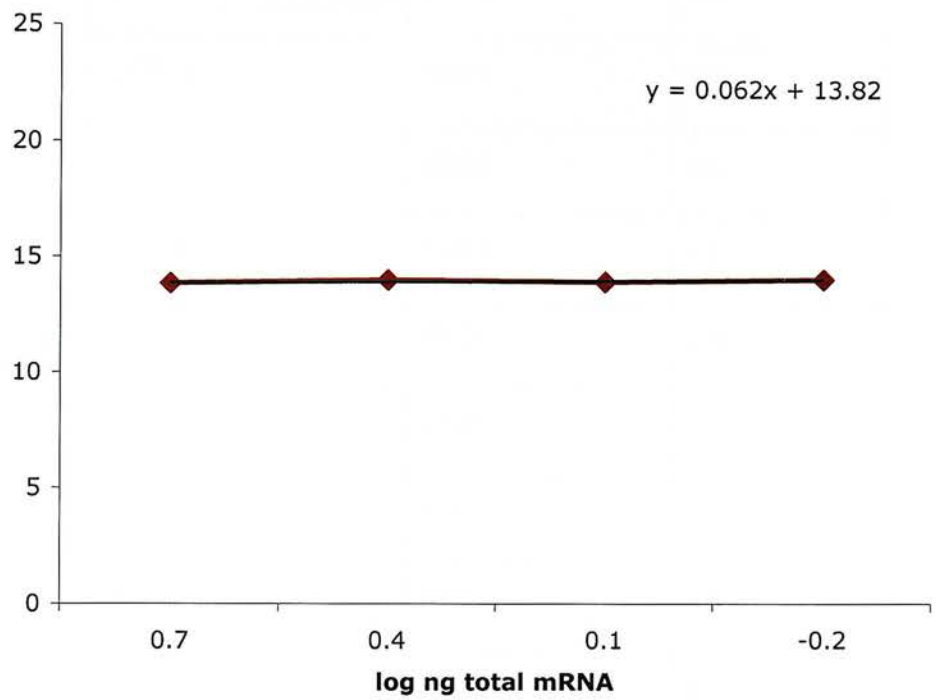


Figure 2.2

An example of a linearity plot for the primer and probes used for Q RT-PCR. This figure is the plot for the prolactin primer and probes. The y value for this particular plot is 0.062 and is an indication of the linearity.

Primer and Probe set	Linearity (y value)	Precision (%)
CD40L	0.057	7.3
COX-1	0.053	1.6
COX-2	0.083	2.0
EP2 Receptor	0.045	1.5
EP4 Receptor	0.019	1.0
IFN-γ	0.081	2.4
IGFBP-1	0.099	6.4
IL-15	0.072	2.9
PDE3a	0.06	0.6
PDE4b	-0.001	4.2
PGTP	0.073	1.3
Progesterone Receptor (nuclear)	0.004	0.8
Prolactin	0.062	2.4

Table 2.3

Validation and precision values for the primer and probe sets used in the present study.

To ensure that no genomic DNA contamination was present in the mRNA samples, the β-actin level was measured in the controls from each experiment and in all the RNA samples used for analysis across the menstrual cycle. The samples were not processed for the reverse transcription stage and were measured directly by Q RT-PCR in place of adding cDNA. An arbitrary level of 27, which was 3 standard deviations away from the mean of all samples, was set and any endometrial samples falling below this level were excluded from analysis. One sample was excluded for this reason. Figure 2.3 represents the β-actin measurements for the samples included in this study.

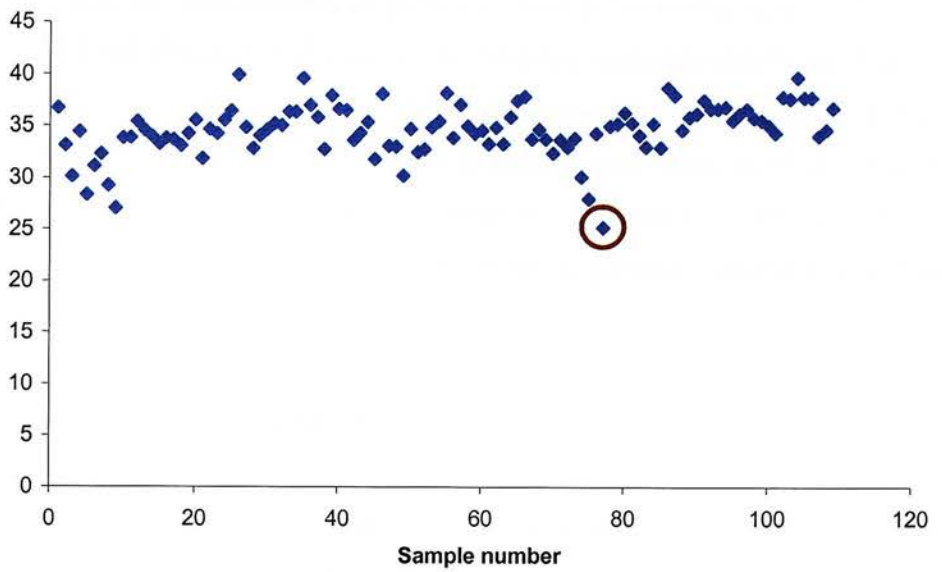


Figure 2.3

The β-actin levels for the experimental control mRNA samples and for the samples used in across the menstrual cycle analyses. The cycle number represents the fluorescence threshold level for all of the samples. Any samples with a value below 27 were excluded from the study. Therefore, the sample circled in red was omitted.

2.6 Enzyme-linked Immunoabsorbant Assay (ELISA)

An enzyme-linked immunoabsorbant assay, an ELISA, can be used to detect and measure various proteins released by cells during *in vitro* culture. The media in which the cells have been grown and treated was collected and stored at -20°C. The ELISA detects the amount of a particular protein present and measures this against a standard curve to give the concentration of protein present in the media sample. There are two types of ELISA that will be discussed: the Two-site sandwich ELISA (section 2.6.1) and the Competition ELISA (section 2.6.2). Both types are run on Maxisorp 96-well plates (Nunc, Denmark). Method files for each ELISA were constructed in Assay Zap and contained information concerning the range of standards used and the level of non-specific binding. This allows a standard curve to be produced against which the samples can be measured against.

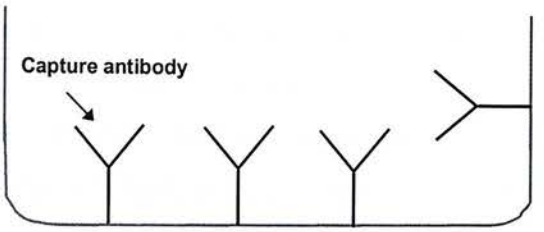
2.6.1 Two-site Sandwich ELSIA

Prior to the assay, plates are coated with a capture monoclonal antibody that is specific against the protein of interest. Blocking and protecting medium (2% Polyvinyl Pyrollidone; 5mg/ml BSA; 0.5ml/litre Preservatives (200mg/ml 5-bromo-5-nitro-1, 3-dione and 200mg/ml 2-methyl-4-isothiazolin-3-one in DMF/DMSO 1:2); 5mM EDTA; 50mM EDTA) is added to prevent non-specific binding and reduce background recordings across all samples. The samples are incubated with the standards, quality controls (of a set concentration in multiple replicates), non-specific binding wells and finally the media samples to be assayed. Any free, specific protein in the wells will spontaneously bind to the capture antibody on the base and sides of the well. Excess media is discarded and washed away before a biotinylated detection antibody (secondary) is added. This will bind to the protein already bound on the plate and this is detected with streptavidin peroxidase conjugate (SPC), diluted in assay buffer (see specific assays for details). After washing the plates once more to remove any unbound SPC, substrate is added. The peroxidase substrate consisted of 20ml sodium acetate

(pH 6; 100mM), 2ml 0.5% urea hydrogen peroxidase (pH 6) and 2ml tetramethyl benzidine (3mg/ml). Any bound peroxidase will metabolise the hydrogen peroxide present in the substrate and the tetra methyl benzidine will be converted into a coloured product. The reaction is stopped by addition of 2N sulphuric acid. The colour is then measured on a plate reader at 450nM. The details of this ELISA are summarised in figure 2.4.

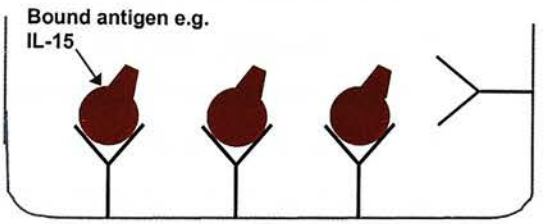
Stage 1:

The capture antibody is bound to the base and sides of the wells.



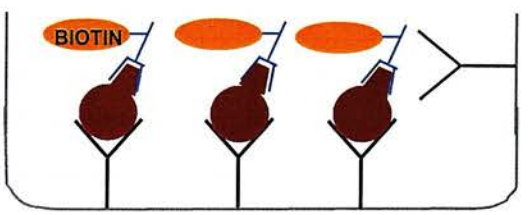
Stage 2:

Any antigens in the media specific to the capture antibody bind.



Stage 3:

A biotin-labelled detection antibody is added and it binds to the bound antigen.



Stage 4:

Streptavidin peroxidase conjugate is added to the wells and this converts the substrate to a coloured product that can be measured.

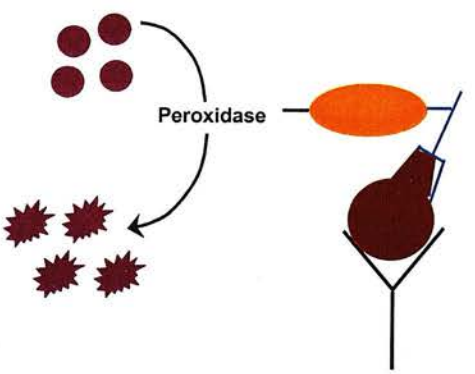


Figure 2.4

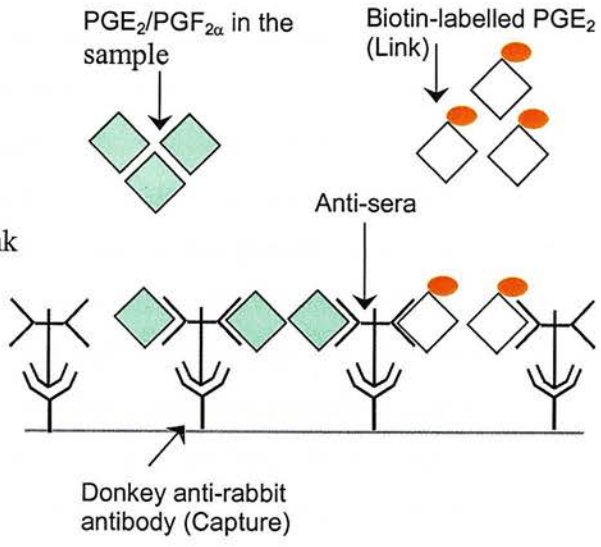
Description of the stages and principles of a two-site sandwich ELISA.

2.6.2 Competition ELISA ($PGF_{2\alpha}$ and PGE_2)

A Competition ELISA (see figure 2.5) works on a different basis to the Two-site sandwich ELISA. Initially plates are coated with a purified donkey anti-rabbit antibody (Sapu, Scotland, UK). Blocking and protecting medium is added to block any non-specific binding and reduce background. For the PGE_2 ELISA, a PGE_2 anti-sera (AS) raised in rabbit is added to the wells and this will bind to PGE_2. Since the coating antibody is anti-rabbit, this will also bind with the AS. Biotinylated labelled PGE_2 is added at the same time and this is referred to as the Link. Samples are added in this same step and this allows the PGE_2 protein in the sample to compete with the Link for binding sites on the AS. As in other ELISAs, a standard curve and quality control wells are added to the plate. The standard contains unlabelled PGE_2 that will compete for biding sites on the AS in the same way as for the unknown samples. The maximum binding is measured by addition of the Link and AS in duplicate wells and this is referred to as the B_0 value. The assay works on the principle that the greater the level of PGE_2 in the sample the greater the amount of displacement of the Link. This results in lower streptavidin binding and thus less colour product in the wells.

Stage 1:

The $PGE_2/PGF_{2\alpha}$ in the sample competes with the biotin-labelled link for anti-sera biding sites.



Stage 2:

The streptavidin peroxidase binds to the biotin on the bound link and converts the substrate into a coloured product that can be measured. The greater the level of $PGE_2/PGF_{2\alpha}$ in the sample, the lower the concentration of coloured product.

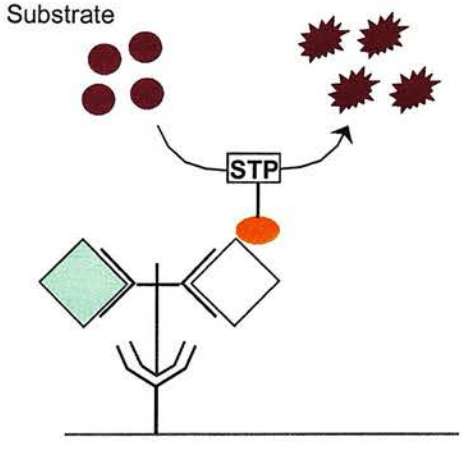


Figure 2.5

Description of the stages and the principles of a Competition ELISA.

2.7 Immunohistochemistry

The immunohistochemical method used was a standard avidin-biotin peroxidase (ABC) method allowing specific proteins within formalin fixed and paraffin embedded endometrial tissue sections to be detected and visualised. Avidin and biotin have a high affinity for one another and it is this property that is the basis of the method. A primary antibody is added and will bind specifically to the protein of interest in the tissue section. A biotinylated secondary antibody that is specific to the species in which the primary antibody was raised is added and will thus bind to the primary antibody. Addition of an avidin-biotin and horseradish peroxidase enzyme complex will result in any free sites on the avidin molecule binding to the biotin on the secondary antibody. When 3,3 diaminobenzidine (DAB) is added it becomes oxidised the resulting colour change of the chromagen causes an insoluble brown precipitate to form at the site of the protein of interest.

2.7.1 Preparation

Tissue sections and cultured cells embedded in agarose and then in paraffin wax were dewaxed in histoclear for 5 minutes. Slides were transferred into a second vial of histoclear for a further 5 minutes. The sections were dehydrated in an alcohol series starting in absolute ethanol for 20 seconds, then into 95% ethanol and finally 70% ethanol, each for 20 seconds. Slides were transferred into dH_2O and washed once in PBS.

2.7.2 Scoring of Immunohistochemistry

A semi-quantitative scoring system was used to measure the location and intensity immunostaining. The sections were scored blind and individually by two observers. The following system was employed: 0 = no immunoreactivity; 1 = faint immunoreactivity; 2 = moderate immunoreactivity; 3 = strong immunoreactivity. All of the slides analysed were included in the same immunohistochemical run and therefore a

fair comparison could be made between them. Initially all of the slides were examined briefly to assess the extremes in staining intensity. The staining intensity and area of staining across each slide was considered as a whole before deciding on the score value to give it. The two observers then came together and compared the scores they had asigned. If there were any discrepancies the slides were re-examined and a mutal decision made on the score that should be given.

2.8 Statistical Analysis

Significant difference of the Q RT-PCR and ELISA data was determined by analysis of variance (ANOVA) using the computer package Statview 3.0. Individual differences were assigned using Fisher's protected least square differences (PLSD) test.

The IL-15 immunohistochemistry results were analysed by Kruskal-Wallis analysis and Dunn's Multiple Comparisons Test used to assign significance (Instat 2.03). This approach was necessary since the data is non-continuous.

3. Features of Endometrial Decidualisation – *in vitro* studies

3.1 Introduction

The sequential development of the human endometrium is a prerequisite for successful implantation and continued pregnancy (Wilcox *et al.*, 1999; Lessey, 2000). In humans, the stromal compartment of the endometrium experiences a transformation across the mid- and late-secretory phases of the menstrual cycle referred to as decidualisation. This begins within the perivascular stromal cells and spreads throughout the stromal region contributing to the formation of decidua in pregnancy. The cells become larger and more rounded in appearance (figure 3.1) with intracellular structural rearrangements and increasing stromal oedema. The major *in vivo* trigger of these adaptations is considered to be progesterone acting upon oestrogen-primed cells (Milligan *et al.*, 1995; King, 2000). The differentiation of these cells runs parallel with the rise in progesterone levels across the secretory phase. Studies *in vitro* on primary cultures of human endometrial stromal cells (ESCs) have revealed that this process is complex implicating the involvement of other factors such as PGE_2 (Frank *et al.*, 1994), relaxin (Tabanelli *et al.*, 1992; Lane *et al.*, 1994) and cAMP (Tang *et al.*, 1993a; Yee *et al.*, 1993; Brar *et al.*, 1997) in addition to progesterone. More recently, Activin A, a member of the TGF-β superfamily, has been implicated as another inducer of decidualisation in ESC at least *in vitro* (Jones *et al.*, 2002) (Figure 3.1).

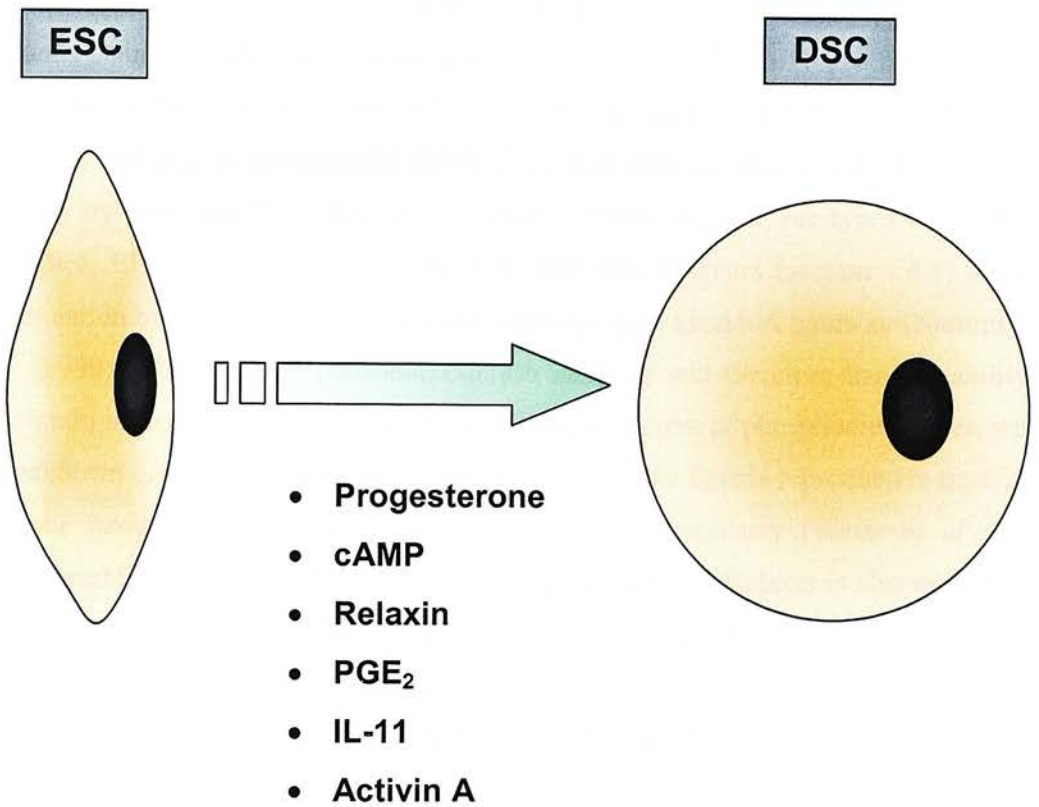


Figure 3.1

Diagrammatic illustration of the various factors thought to be involved in decidualisation of human ESCs to decidualised stromal cells (DSC), highlighting the multi-factorial nature of the process.

PGs are synthesised from the intermediate, PGH_2, (see sections 1.4.1 and 2.1 for details) by specific PG synthases (Smith *et al.*, 1996; Smith *et al.*, 1996; Murakami *et al.*, 2003). PGE_2 and PGE_2 synthase are located in glandular, stromal and epithelial cells in the functional layer of the human endometrium at all cycle stages with a reduction in stromal staining in the late secretory phase (Milne *et al.*, 2001). Prostaglandins may be important in the secretory phase and specifically during the implantation window since the EP2 receptor is up-regulated 4-fold compared with the late proliferative phase as shown by gene profiling (Kao *et al.*, 2002). There are four sub-types of the PGE_2 receptor, EP1-4 and activation of the EP2 and EP4 receptors (section 1.4.1) leads to stimulation of adenylate cyclase and the cAMP/protein kinase A pathway (Narumiya *et al.*, 1999). Relaxin is a phosphodiesterase inhibitor and therefore has the ability to maintain intracellular cAMP levels by inhibiting the action of phosphodiesterases, which breakdown cAMP. The principal source of relaxin in the female reproductive tract is the corpus luteum in the luteal phase and throughout pregnancy (Weiss *et al.*, 1978; Blankenship *et al.*, 1994; Bani, 1997; Sunder *et al.*, 2000). Relaxin is also expressed in the endometrium and decidua (Bryant-Greenwood *et al.*, 1993). Epithelial and stromal cells have been reported to display immuno-reactivity for relaxin with an increase in intensity across the secretory phase and throughout pregnancy.

It is not only the morphology of the stromal cells that transforms with decidualisation, but also their function. The stroma provides more extensive functions than a mere supportive structural role. It is believed that decidualised stromal cells are important in providing homeostasis during implantation and trophoblast invasion (Lockwood *et al.*, 1999). Progesterone receptor knock-out mice fail to exhibit decidualisation and this is associated with implantation failure (Rider, 2002). Trophoblast invasion in humans is significantly deeper in areas where the uterus is deficient in decidua caused by scar tissue. Imbalances in implantation and placentation can result in uterine rupture or the later development of pre-eclampsia (Loke *et al.*, 1997) highlighting the necessity for tight regulation of these events. The depth of trophoblast invasion in humans is under the influence of hCG, progesterone and oestradiol (Mitreski *et al.*, 2003) and in ectopic

pregnancies, the extent of trophoblast infiltration has also been shown to be influenced by hCG levels (Natale *et al.*, 2003). In humans, whether the process of decidualisation is irreversible is a matter for discussion and will be addressed in this chapter. Relaxin has previously been shown to acutely and permanently raise intracellular cAMP levels in cultured ESCs, resulting in prolactin secretion (Telgmann *et al.*, 1997).

Documented markers of the decidualisation process include prolactin (Maslar *et al.*, 1979; Tseng *et al.*, 1999) and IGFBP-1 (Bell *et al.*, 1991; Bryant-Greenwood *et al.*, 1993; Lee *et al.*, 1997) and these can be used as guidelines to observe and identify decidualisation *in vitro*. In humans, prolactin levels start to increase in early secretory phase endometrial stroma reaching maximal levels in decidua as shown by immunohistochemistry (Bryant-Greenwood *et al.*, 1993). This study also revealed a rise in IGFBP-1 immuno staining with a different timing of appearance to that of prolactin. IGFBP-1 was absent from proliferative and early secretory phase endometrium but began to appear in the mid secretory phase followed by a dramatic rise in the late secretory stroma (Figure 3.2). Other markers of decidualisation that have been identified in human and rat decidual stroma include vimentin, desmin, laminin and fibronectin (Glasser *et al.*, 1986; Glasser *et al.*, 1987; Van Muijen *et al.*, 1987) and these represent structural rearrangements within the cells.

The number of uNK cells present in the human endometrium increases across the secretory phase, particularly in the mid-secretory phase. This implies hormonal control over these cells (King *et al.*, 1989b) although they do not express the nuclear progesterone receptor (King *et al.*, 1996; Henderson *et al.*, 2003). Studies on uNK cells have demonstrated the ability of the cytokine IL-15 to stimulate proliferation of these cells *in vitro* (Verma *et al.*, 2000). A relationship between IL-15 and progesterone-induced decidualisation of human ESCs *in vitro* has been proposed (Okada *et al.*, 2000a). This provides a link between the functions of ESCs and uNK cells within the endometrium that may be important to successful embryo implantation and early pregnancy maintenance.

This chapter examines decidualisation in ESC cultures *in vitro*, studying the effects of treatment with a synthetic progestin, PGE_2 and 8-Bromo cAMP on the expression and release of prolactin and IGFBP-1. Using these data as an indication of decidualisation in these cultures a comparison between IL-15 mRNA expression and decidualisation *in vitro* has been made. Levels of the genomic progesterone receptor expression in these cells have been established. The effect of withdrawing decidualisation treatment on prolactin expression is also examined.

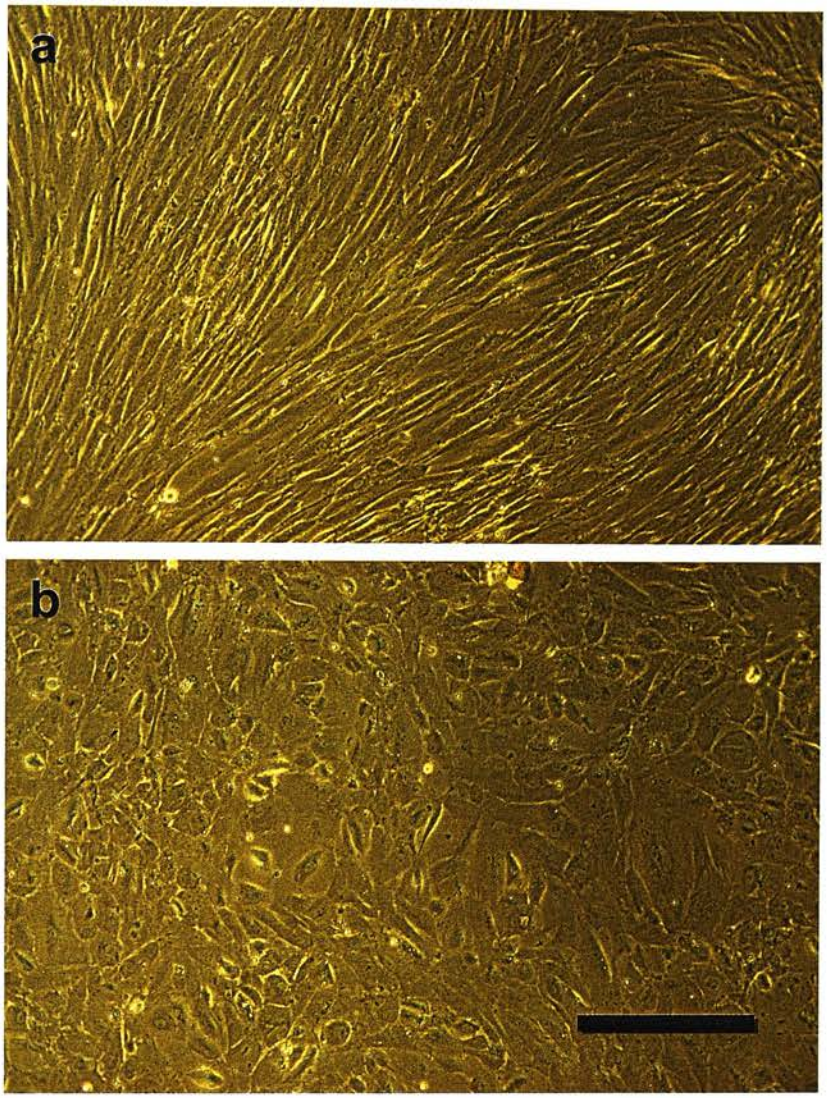


Figure 3.2

Photomicrographs of non-decidualised ESC (**a**) and decidualised ESC (**b**) primary cultures. The decidualised ESCs were treated for 6 days with a synthetic progestin, MPA, (10^{-6} M) plus 8-Bromo cAMP (250μM) and have become much rounder in appearance compared with the non-decidualised ESCs. Scale bar represents 300μm.

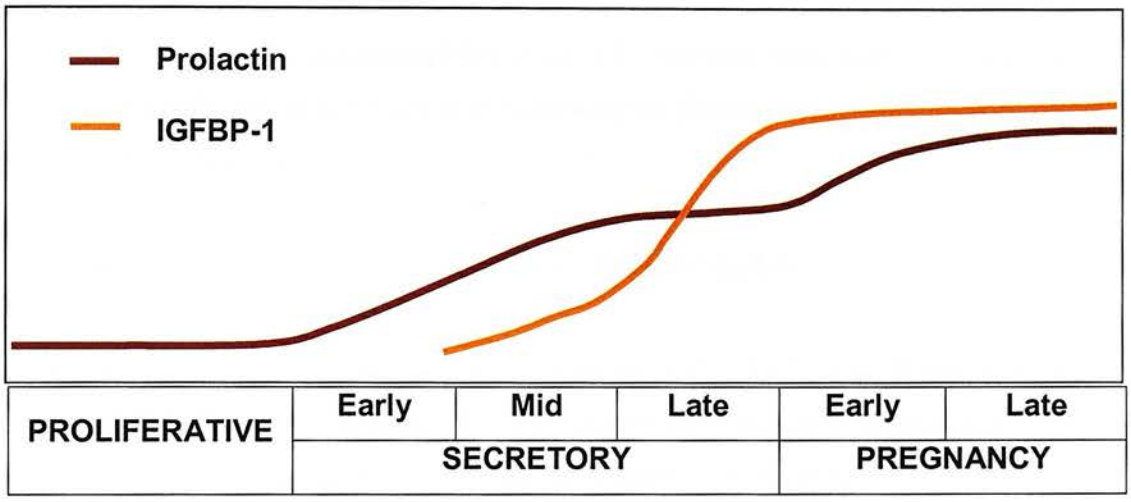


Figure 3.3

Schematic diagram of the variation in stromal immuno staining intensity of prolactin and IGFBP-1 across the human menstrual cycle and throughout pregnancy. Based on data from Bryant-Greenwood *et al* 1993 (Bryant-Greenwood *et al.*, 1993).

3.2 Materials and Methods

3.2.1 Human Uterine Tissue Collection

Proliferative (n = 5) and early secretory (n = 4) endometrial biopsies were collected and processed as described earlier (section 2.1). All samples were processed to separate the ESCs from the glands, as described in section 2.2. The cells were grown for a period of two weeks prior to commencing experiments in the presence of Medroxyprogesterone acetate (MPA) and bFGF.

3.2.2 *in vitro* Primary Cell Culture Studies – Decidualisation

ESCs were seeded in 12-well plates at a concentration of $1.4x10^5$ cells/ml and allowed to adhere overnight. The following treatment regimes were employed as detailed in tables 3.2 and 3.3. The experiments were designed to study *in vitro* decidualisation of ESCs. MPA is a synthetic progestin and is relatively stable. It was selected as a treatment to analyse the effects of a progestin alone on *in vitro* decidualisation. Both PGE_2 and synthetic cAMP analogues have previously been acknowledged as stimulators of decidualisation *in vitro* and were therefore included in the treatment regime (see section 3.1). 8-Bromo cAMP is a cAMP analogue and was selected because it is readily absorbed by cells and partially resistant to phosphodiesterases. In addition, the combined effects of MPA plus PGE_2 or 8-Bromo cAMP were assessed. The experiment detailed in table 3.3 was designed to determine whether a continuous supply of treatments was required to sustain decidualisation in ESCs *in vitro*. Relative levels of prolactin mRNA will be analysed to assess this (see 3.2.5).

Table 3.2

Experiment 1: Decidualisation of ESCs (n = 4)		
Treatment	**Concentration**	**Incubation time**
Control	N/A	4, 8 and 12 days
MPA	10^{-6} M	
PGE_2	10^{-6} M	
PGE_2 + MPA	10^{-6} M + 10^{-6} M	
8-Bromo cAMP	250μM	
8-Bromo cAMP + MPA	250μM + 10^{-6} M	

Table 3.3

Experiment 2: Decidualisation of ESCs and treatment removal (n = 5)		
Treatment	**Concentration**	**Incubation time**
Control	N/A	5 days, 5 days followed by 5 days with treatment withdrawal, 10 days continuous treatment
PGE_2 + MPA	10^{-6} M + 10^{-6} M	
8-Bromo cAMP + MPA	250μM + 10^{-6} M	

3.2.3 Prolactin ELISA

Media collected from experiment 1 at the termination of the time-points, (4, 8 and 12 days), was assayed on a commercial Wallac DELFIA® Prolactin kit (PerkinElmer™ Life Sciences, Wallac Oy, Turku, Finalnd). The kit instructions were adhered to with the exception that 150μl of sample media was added in place of the suggested 25μl to increase the sensitivity of the assay.

3.2.4 IGFBP-1 ELISA

Media collected from experiment 1 at the termination of the time-points, (4, 8 and 12 days), was assayed for IGFBP-1 using a two-site sandwich ELISA (section 2.6.1) (R & D Systems). Plates were coated with the IGFBP-1 capture antibody and incubated according to the IL-15 ELISA method. The antibody was added at 2µg/ml and 100µl per well. The plates were washed prior to use and a standard curve added in single wells to each plate consisting of eight standards starting with a top standard of 8ng/ml. 1:2 dilutions were performed until the bottom standard of 0.06ng/ml was obtained. Buffer plus Tween (B+T) was used to dilute samples and NSB wells were added as 100µl of B+T. A quality control was made up at 700pg/ml from the IGFBP-1 standard and added in quadruplicate wells to each plate. All media samples to be tested were added in quadruplicate wells, 100µl per well. Plates were sealed and incubated for 2 hours on a plate shaker at room temperature. The plates were washed in wash buffer four times each and tapped dry. The IGFBP-1 detection antibody was then added at 0.1µg/ml and 100µl per well and incubated at room temperature for 2 hours on a plate shaker. Plates were washed as before and tapped dry. Streptavidin peroxidase conjugate (Roche) was diluted in B+T to 0.125U/ml and 100µl pipetted into all wells. Plates were sealed and incubated for 20 minutes on a plate shaker at room temperature. The plates were then washed as before and 100µl of substrate added per well. Plates were allowed to develop for 15 minutes before stopping the reaction with 50µl of 2N sulphuric acid per well. All plates were read on a plate reader at 450nM. The computer program Assay Zap was used to analyse the results and construct a standard curve against which the samples could be judged.

3.2.5 RNA Extraction and Q RT-PCR

The RNA was extracted and cDNA prepared from the ESC experiments 1 and 2. Prolactin, IGFBP-1, IL-15 and progesterone receptor mRNA levels were measured by Q RT-PCR as described in section 2.5.

3.2.6 Statistical Analysis

Q RT-PCR and ELISA data were analysed by the methods described previously in section 2.8.

3.3 Results

3.3.1 Decidualisation of ESC Studies

3.3.1a Prolactin mRNA Expression and Release by ESCs

The mRNA expression of prolactin after 4 days is almost 4-fold greater than controls with MPA or with 8-Bromo cAMP treatment. Treatment with a combination of MPA and 8-Bromo cAMP stimulated a 22-fold increase in prolactin mRNA levels ($P < 0.017$). Prolactin protein released into the media after 4 days was below the minimum detection limit (250pg/ml) of the ELISA. After 8 days of treatment the combined treatment of PGE_2 and MPA had caused a 40-fold rise in prolactin mRNA relative to controls. A 163-fold increase in prolactin mRNA was produced by treatment with 8-Bromo-cAMP and MPA in combination ($P < 0.006$). This was comparable with the ELISA data that recorded 3-fold and 9-fold increases in prolactin release with PGE_2 plus MPA and 8-Bromo-cAMP plus MPA ($P < 0.009$) treatment respectively. The same pattern of prolactin mRNA expression and protein release were observed after 12 days of treatment. PGE_2 plus MPA stimulated an 11-fold rise in mRNA and a 9-fold increase in protein levels. 8-Bromo-cAMP plus MPA triggered a 29-fold increase in prolactin mRNA levels ($P < 0.03$) and a 22-fold increase in protein release ($P < 0.028$). Modest rises in prolactin mRNA expression and release were produced by treatment with 8-Bromo cAMP alone.

Figure 3.3

Relative levels of prolactin mRNA expression after 4 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **a** $p < 0.017$.

Figure 3.4

Relative levels of prolactin mRNA expression after 8 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **b** $p < 0.006$.

Figure 3.5

Relative levels of prolactin mRNA expression 12 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **c** $p < 0.03$.

Figure 3.3

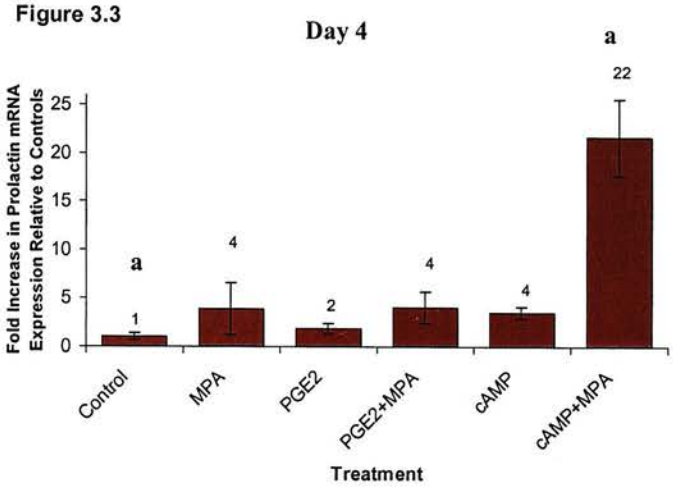


Figure 3.4

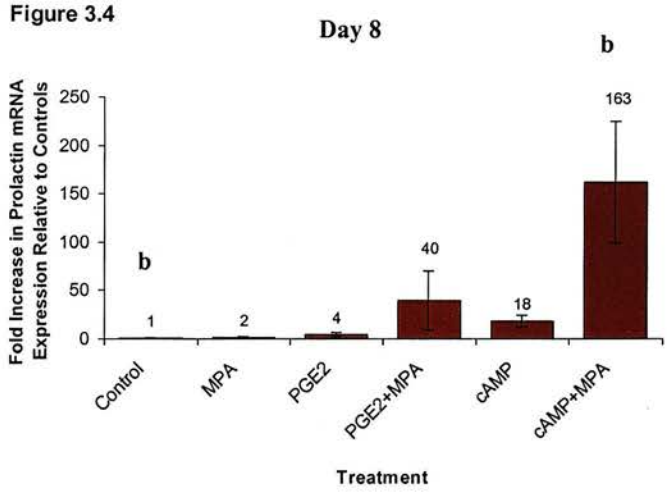


Figure 3.5

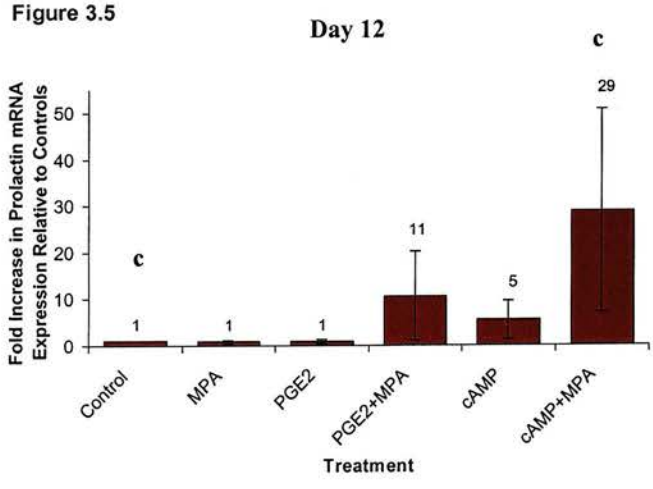


Figure 3.6

Prolactin protein release by human ESCs over 48 hours after 8 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. Same letters denote significant difference. **a** $p < 0.009$. The lower detection limit of the ELISA was 0.25ng/ml.

Figure 3.7

Prolactin protein release by human ESCs over 48 hours after 12 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. Same letters denote significant difference. **b** $p < 0.028$. The lower detection limit of the ELISA was 0.25ng/ml.

Figure 3.6

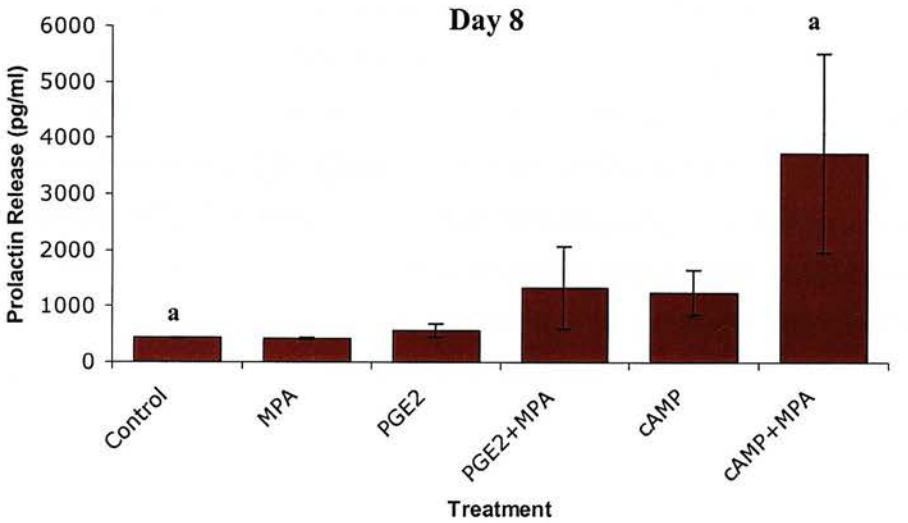
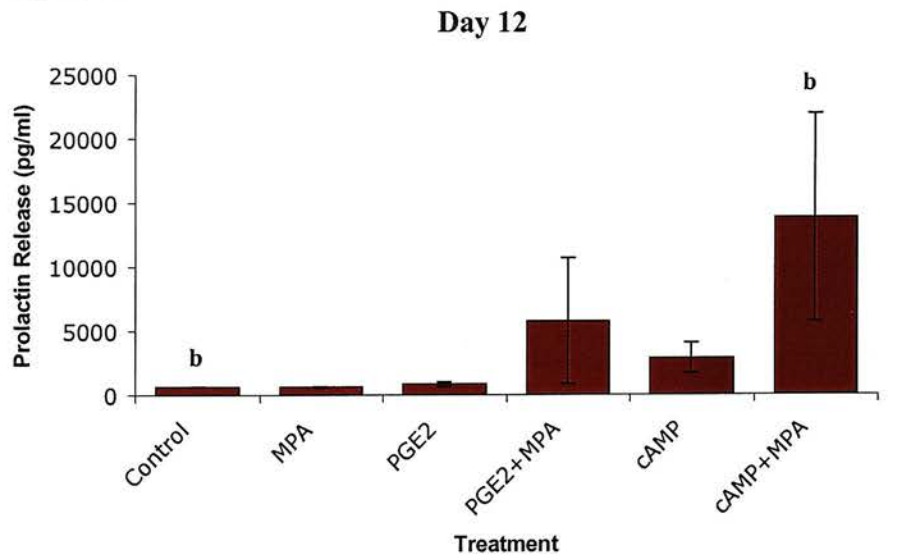


Figure 3.7



3.3.1b IGFBP-1 mRNA Expression and Release by ESCs

The mRNA expression for IGFBP-1 was increased considerably by treatment of ESCs with PGE_2 plus MPA (1766-fold increase) and 8-Bromo cAMP plus MPA (9075-fold increase) ($P < 0.019$) after 4 days. Treatment with PGE_2 alone resulted in a 32-fold rise in IGFBP-1 mRNA expression relative to controls. 8-Bromo cAMP promoted a fold increase of approximately 800. These results were consistent with the IGFBP-1 ELISA data, illustrating a 25-fold increase in protein release on day 4 with PGE_2 plus MPA treatment and an 85-fold increase following incubation with 8-Bromo-cAMP plus MPA ($P < 0.034$). After 8 and 12 days of incubation with the treatments corresponding patterns in mRNA expression and release were observed.

Figure 3.8

Relative levels of IGFBP-1 mRNA expression after 4 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **a** $p < 0.019$

Figure 3.9

Relative levels of IGFBP-1 mRNA expression after 8 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **b** $p < 0.015$,

Figure 3.10

Relative levels of IGFBP-1 mRNA expression after 12 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **c** $p < 0.017$.

Figure 3.8

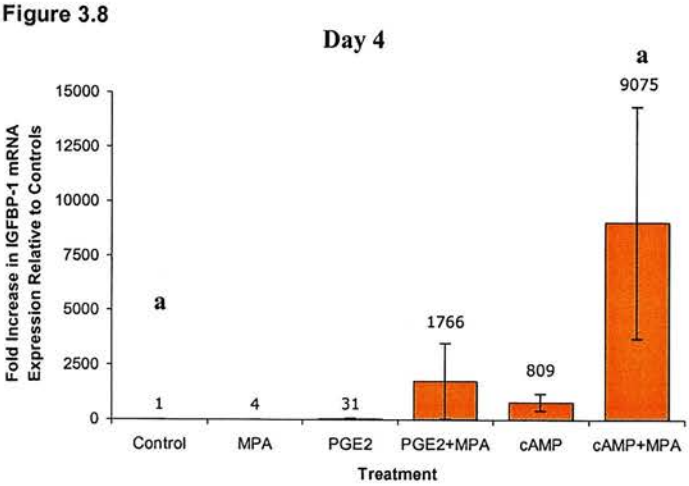


Figure 3.9

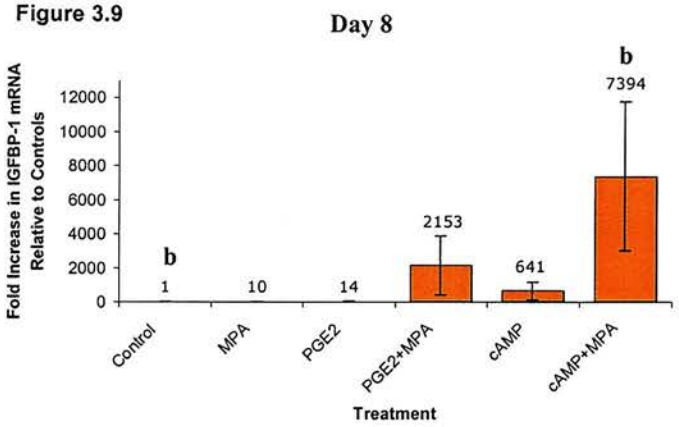
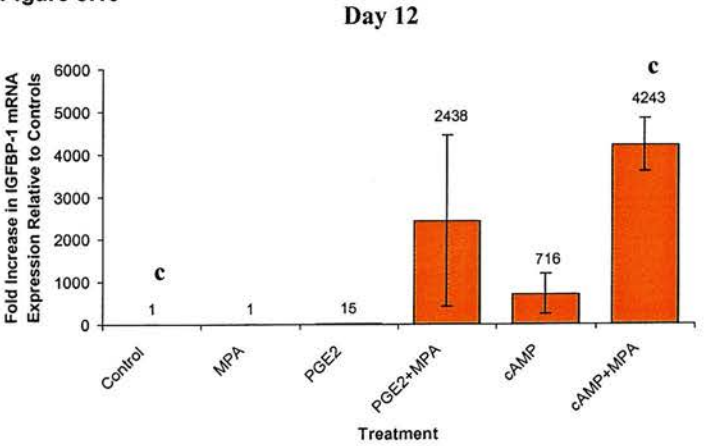


Figure 3.10



Figures 3.11

IGFBP-1 protein release (pg/ml) by human ESCs over 48 hours after 4 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. Same letters denote significant difference. **a** $p < 0.034$. The lower detection limit of the ELISA was 60pg/ml.

Figure 3.12

IGFBP-1 protein release (pg/ml) by human ESCs over 48 hours after 8 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. Same letters denote significant difference. **b** $p < 0.011$. The lower detection limit of the ELISA was 60pg/ml.

Figure 3.13

IGFBP-1 protein release (pg/ml) by human ESCs over 48 hours after 12 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. Same letters denote significant difference. **c** $p < 0.034$. The lower detection limit of the ELISA was 60pg/ml.

Figure 3.11

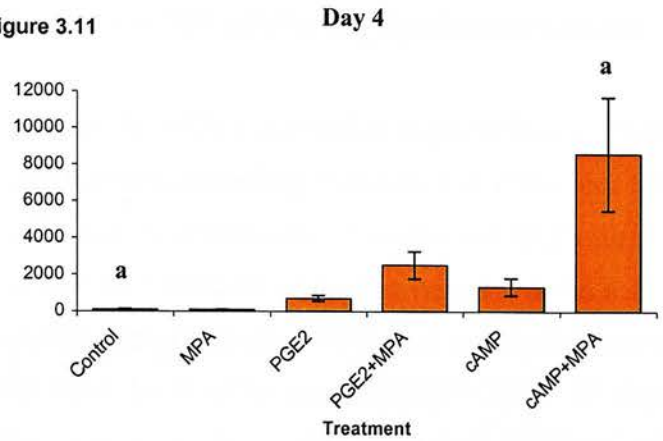


Figure 3.12

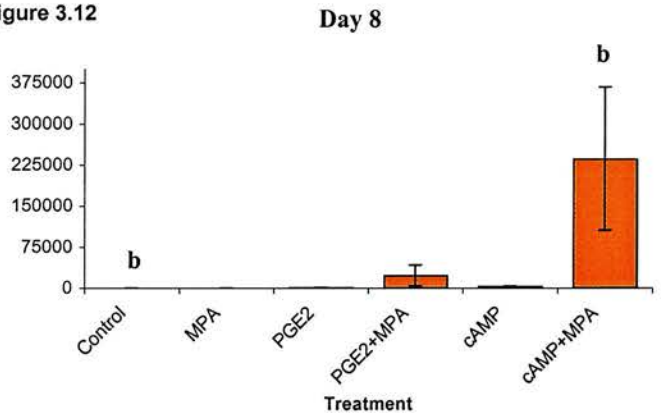
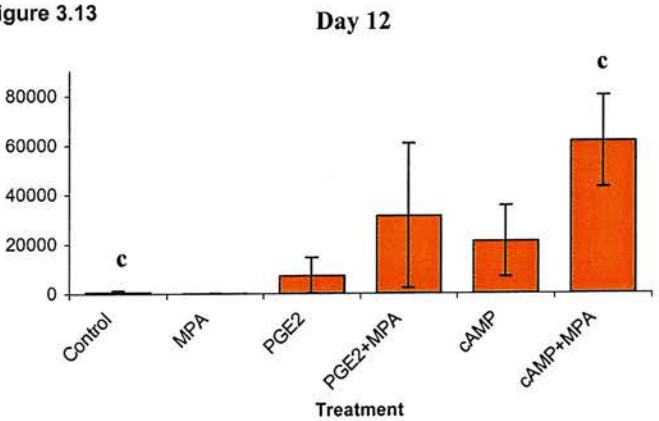


Figure 3.13



3.3.2 Decidualisation State of ESCs Following Treatment Removal

After 5 days of treatment the mRNA expression of prolactin was 77-fold and 261-fold greater compared with controls proceeding treatment with PGE_2 plus MPA and 8-Bromo cAMP plus MPA ($P < 0.017$) respectively. Treatment of ESCs for 5 days and then a further 5 days of culture with removal of treatments gave rise to a 44-fold increase in those initially incubated with PGE_2 plus MPA and a 139-fold increase following 8-Bromo cAMP plus MPA ($p < 0.04$) pre-treatment. After 10 days of continuous incubation with the treatments, PGE_2 plus MPA and 8-Bromo cAMP plus MPA stimulated 566- and 1187-fold ($p < 0.019$) rises in prolactin mRNA levels relative to controls.

Figure 3.14

Relative levels of prolactin (prl) mRNA expressed in ESCs treated for 5 days with decidualising stimuli. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **a** $p < 0.017$.

Figure 3.15

Relative levels of prolactin (prl) mRNA expressed in ESCs treated for 5 days with decidualising stimuli followed by 5 days of treatment withdrawal. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **b** $p < 0.04$.

Figure 3.16

Relative levels of prolactin (prl) mRNA expressed in ESCs treated for 10 days with decidualising stimuli. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **c** $p < 0.019$.

Figure 3.14

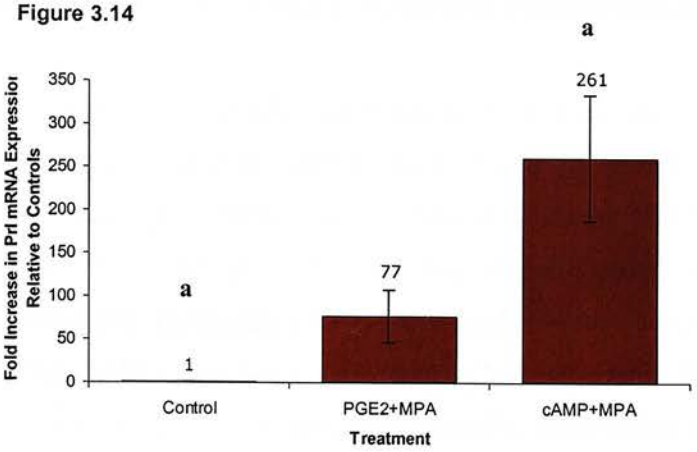


Figure 3.15

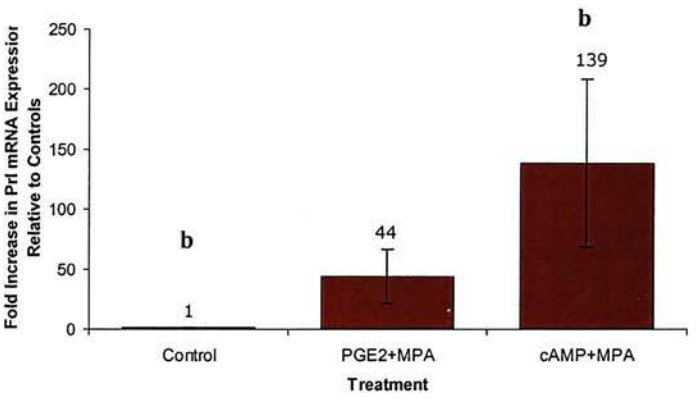
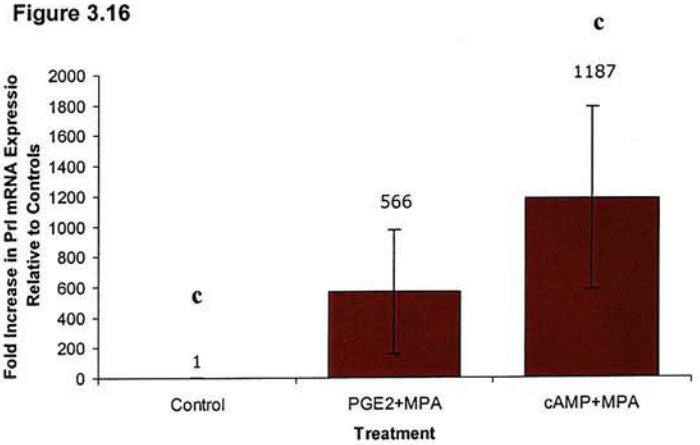


Figure 3.16



3.3.3 IL-15 mRNA Expression in ESCs Treated with Decidualising Stimuli *in vitro*

After 4 days of treatment with PGE_2 plus MPA an 11-fold increase in IL-15 mRNA expression was observed relative to controls and a 22-fold rise in ESCs treated with 8-Bromo cAMP plus MPA ($p < 0.023$). After 8 days of treatment with PGE_2 plus MPA and 8-Bromo cAMP plus MPA ($p < 0.028$) 26- and 60-fold increases in IL-15 mRNA expression were recorded. Following a further 4 days of treatment on day 12, PGE_2 plus MPA and 8-Bromo cAMP plus MPA ($p < 0.028$) had stimulated 20- and 168-fold increases in IL-15 mRNA levels compared with controls. MPA added alone produced a 2-fold increase in expression and 8-Bromo cAMP on its own stimulated 4-, 18- and 25-fold rises in IL-15 mRNA levels on days 4, 8 and 12 respectively.

Figure 3.17

Relative levels of IL-15 mRNA expression after 4 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **a** $p < 0.023$

Figure 3.18

Relative levels of IL-15 mRNA expression after 8 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **b** $p < 0.028$

Figure 3.19

Relative levels of IL-15 mRNA expression after 12 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **c** $p < 0.028$.

Figure 3.17

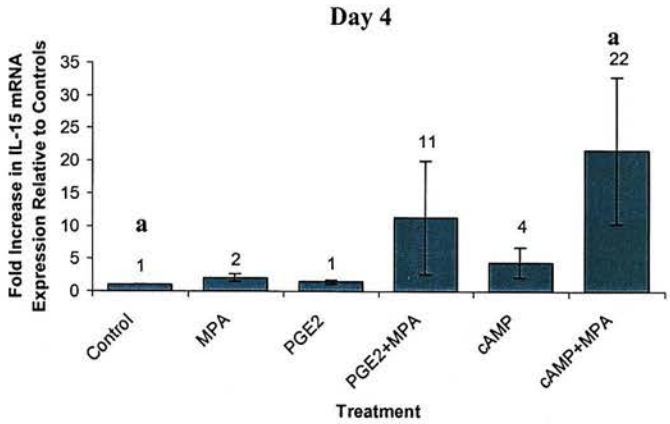


Figure 3.18

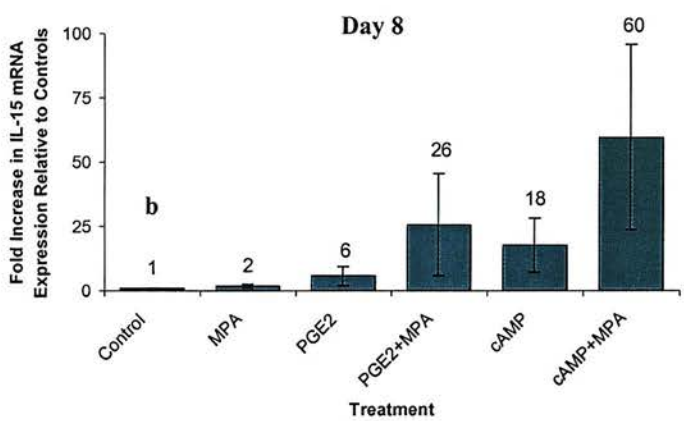


Figure 3.19

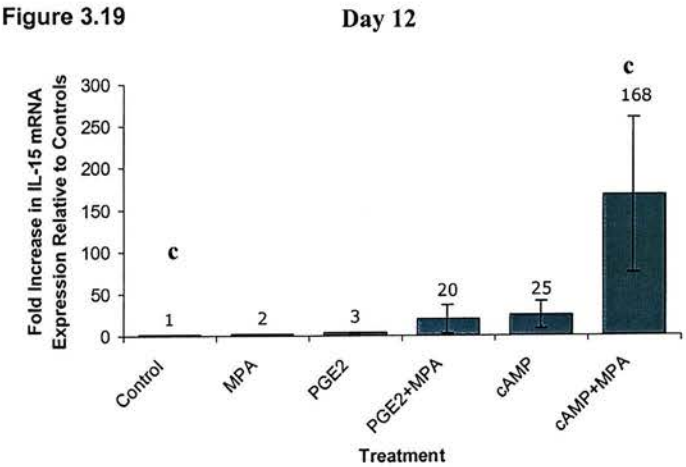


Figure 3.20 Correlation between prolactin and IL-15 mRNA levels on day 4

The correlation coefficient was calculated for the relative expression levels of IL-15 and prolactin mRNA for the different treatments on day 4 and found to be 0.92. This graph represents the log values of prolactin mRNA expression against the log values of IL-15 mRNA expression for the different treatment groups and illustrates the rise in IL-15 expression along with prolactin levels. The R^2 value is stated on the graph.

Figure 3.21 Correlation between prolactin and IL-15 mRNA levels on day 8

The correlation coefficient was calculated for the relative expression levels of IL-15 and prolactin mRNA for the different treatments on day 8 and found to be 0.97. This graph represents the log values of prolactin mRNA expression against the log values of IL-15 mRNA expression for the different treatment groups and illustrates the rise in IL-15 expression along with prolactin levels. The R^2 value is stated on the graph.

Figure 3.22 Correlation between prolactin and IL-15 mRNA levels on day 12

The correlation coefficient was calculated for the relative expression levels of IL-15 and prolactin mRNA for the different treatments on day 12 and found to be 0.97. This graph represents the log values of prolactin mRNA expression against the log values of IL-15 mRNA expression for the different treatment groups and illustrates the rise in IL-15 expression along with prolactin levels. The R^2 value is stated on the graph.

Figure 3.20

Day 4

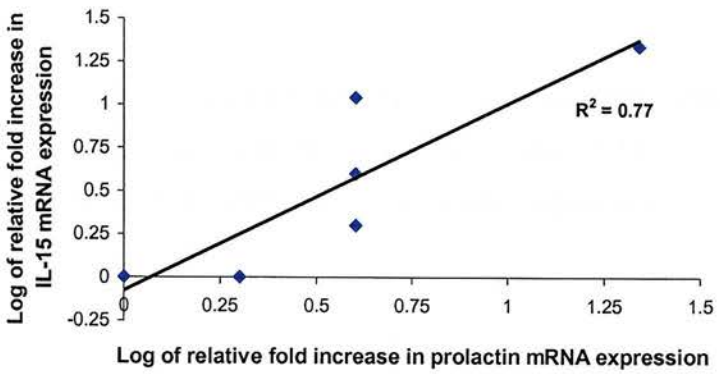


Figure 3.21

Day 8

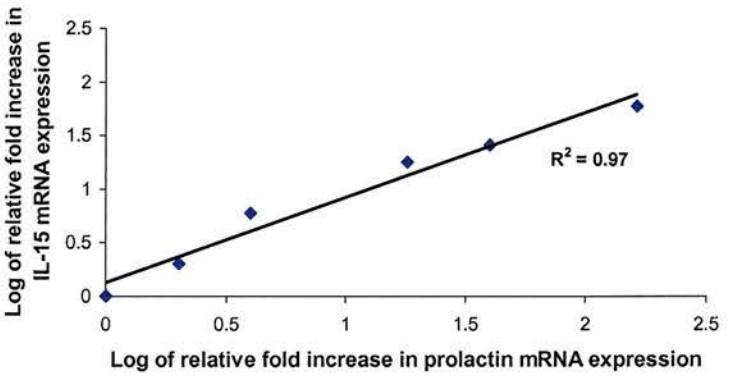
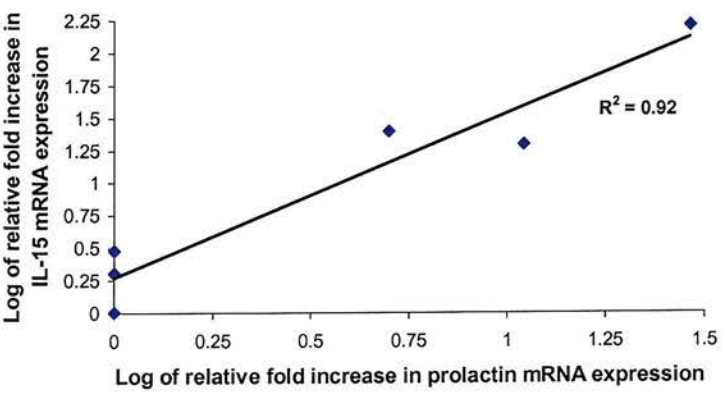


Figure 3.22

Day 12



3.3.5 Progesterone Receptor (nuclear) mRNA Expression in ESCs Treated with Decidualising Stimuli *in vitro*

No significant changes in the genomic progesterone receptor (PR) mRNA levels were observed after ESCs were treated for 4, 8 or 12 days (figure 3.23, 3.24 and 3.25). All treatment groups expressed PR mRNA levels similar to controls values.

Figure 3.23

Relative levels of genomic progesterone receptor (PR) mRNA expression after 4 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro.*

Figure 3.24

Relative levels of genomic progesterone receptor (PR) mRNA expression after 8 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro.*

Figure 3.25

Relative levels of genomic progesterone receptor (PR) mRNA expression after 12 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro.*

Figure 3.23

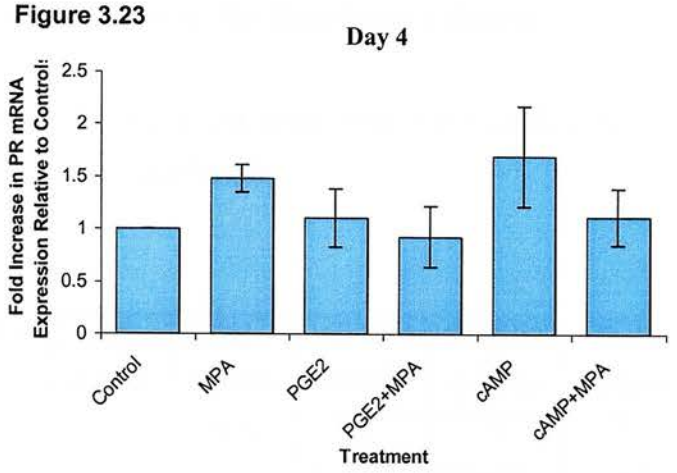


Figure 3.24

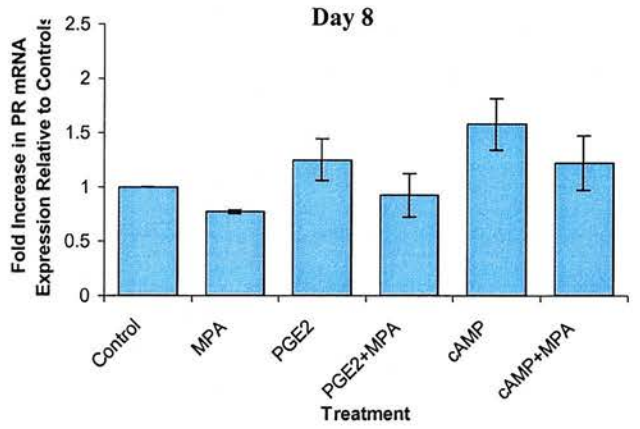


Figure 3.25

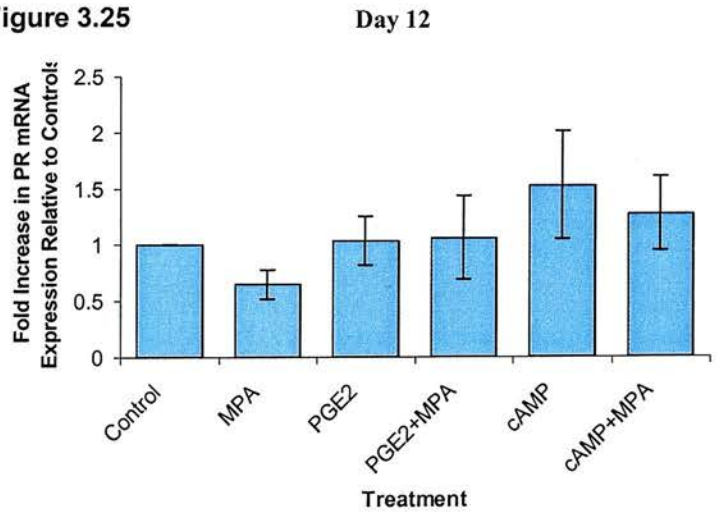


Table 3.4. Summary of Results for Experiment 1 (Day 4)

Numbers represent fold increases in levels relative to controls with significance values given in the brackets were applicable.

Treatment	Prl mRNA	Prl protein	IGFBP-1 mRNA	IGFBP-1 protein	IL-15 mRNA	PR mRNA
Control	1	N/A	1	1	1	1
MPA	4	N/A	4	0.8	2	1.5
PGE_2	2	N/A	31	7	1	1.1
PGE_2 + MPA	4	N/A	1766	25	11	0.9
8-Bromo cAMP	4	N/A	809	13	4	1.7
8-Bromo cAMP + MPA	22 (0.017)	N/A	9075 (0.19)	85 (0.034)	22 (0.023)	1.1

Table 3.5. Summary of results for experiment 1 (Day 8)

Treatment	Prl mRNA	Prl protein	IGFBP-1 mRNA	IGFBP-1 protein	IL-15 mRNA	PR mRNA
Control	1	1	1	1	1	1
MPA	2	1	10	1	2	0.8
PGE_2	4	1.3	14	7	6	1.3
PGE_2 + MPA	40	3.1	2153	299	26	0.9
8-Bromo cAMP	18	2.9	641	39	18	1.6
8-Bromo cAMP + MPA	163 (0.006)	8.7 (0.09)	7394 (0.015)	3081 (0.011)	60 (0.028)	1.3

Table 3.6. Summary of results for experiment 1 (Day 12)

Treatment	Prl mRNA	Prl protein	IGFBP-1 mRNA	IGFBP-1 protein	IL-15 mRNA	PR mRNA
Control	1	1	1	1	1	1
MPA	1	1	1	0.1	2	0.7
PGE_2	1	1.3	15	9	3	1.0
PGE_2 + MPA	11	9.1	2438	39	20	1.1
8-Bromo cAMP	5	4.5	716	26	25	1.5
8-Bromo cAMP + MPA	29 (0.03)	21.8 (0.028)	4243 (0.017)	76 (0.034)	168 (0.028)	1.3

3.4 Discussion

Using an *in vitro* system to study decidualisation in ESCs the results in this chapter have demonstrated the synergistic actions between a synthetic progestin, MPA, and either PGE_2 or 8-Bromo cAMP in inducing prolactin and IGFBP-1 expression and release. These synergistic actions were required for inducing a rise in IL-15 mRNA expression. There was a correlation between the decidualisation status in these cells and IL-15 levels suggesting that IL-15 mRNA levels rise upon decidualisation *in vitro*. Genomic progesterone receptor levels remained consistent between treatment groups and controls. In ESC cultures that were pre-treated for 5 days followed by withdrawal of treatment for 5 days the levels of prolactin remained high in the ESCs treated with MPA and either PGE_2 or 8-Bromo cAMP. These data indicate that over this time period the decidual state of cells remains in place and that suggests a permanent transformation of these cells that cannot be reversed.

In humans, decidualisation occurs in the endometrial stroma during the mid- to late-secretory phases and involves both morphological and biochemical changes in this cell type. This transformation is thought to be essential to embryo implantation and to pregnancy maintenance. Two of the major products of the decidualised stromal cell are IGFBP-1 and prolactin and are believed to have important roles in pregnancy although their specific functions are still not clear. Women with reduced serum IGFBP-1 levels have been shown to have a predisposition to developing pre-eclampsia (Grobman *et al.*, 2001) suggesting a possible role of IGFBP-1 in placentation. Although knock-out mice for the prolactin receptor (Prl-R -/-) show evidence of failed implantation, it was established that the corpus luteum was unable to produce progesterone and administration of a progestin counteracted these defects in the early stages of pregnancy (Binart *et al.*, 2000). However, late pregnancy loss could not be rescued, thereby implicating a discrete function of prolactin in the maintenance of pregnancy in late gestation.

Prolactin and IGFBP-1 are well documented as markers of stromal decidualisation since decidual cells express raised levels versus non-decidualised ESCs (Maslar *et al.*, 1979) (Bryant-Greenwood *et al.*, 1993). The results in this present chapter illustrate further the synergistic ability of cAMP plus a progestin to induce decidualisation *in vitro*. PGE_2 acts via EP2 and EP4 receptors to raise intracellular cAMP and alongside the rising progesterone levels across the secretory phase, this provides one mechanism by which decidualisation can be triggered (figure 3.26). How these separate pathways communicate is unclear. However, experiments using the breast cancer cell line, T47D, have demonstrated a form of cross-talk between cAMP and the progesterone receptor (Edwards *et al.*, 1993). If a similar process occurs in decidualising ESCs this would provide such a link. The results in this chapter showed that no change in the nuclear progesterone receptor mRNA level occurred in concert with decidualisation but this does not exclude the possibility that cAMP is interacting with the progesterone receptor via more discrete mechanisms. In *in vitro* experiments treatment of human ESCs with progesterone plus oestradiol resulted in induction of the EP2 receptor mRNA compared to untreated controls (Brar *et al.*, 1997). It is therefore possible that via the upregulation of this PGE_2 receptor that some of the effects of progesterone may be driven.

The cAMP analogue used in the experiments detailed in this chapter is 8-Bromo cAMP which is absorbed by cells but also resists the destructive actions of phosphodiesterases thus acting as a cAMP mimic. This may explain the potency of this treatment versus PGE_2. Relaxin is a physiological product that acts to raise cAMP levels and has been proposed to be an inhibitor of phosphodiesterases (Bartsch *et al.*, 2001) thus preventing breakdown of intracellular cAMP. It is produced by the corpus luteum (Weiss *et al.*, 1978) and in both secretory endometrium and decidua (Bryant-Greenwood *et al.*, 1993). The possible mechanisms by which progesterone, PGE_2 and relaxin may be converging to induce decidual transformation are illustrated in figure 3.26. In the Hoxa-10 knock-out mouse, defective decidualisation is observed and aberrant expression of the PGE_2 receptors, EP3 and EP4, is apparent (Lim *et al.*, 1999) implicating PGE_2 further in decidual transformation. The timing of appearance of prolactin and IGFBP-1 in the

human uterine stroma as shown by immunohistochemistry is both cyclical and divergent (Bryant-Greenwood *et al.*, 1993) (figure 3.3) and it therefore likely that *in vivo* other mediators in addition to intracellular cAMP levels and progesterone are involved.

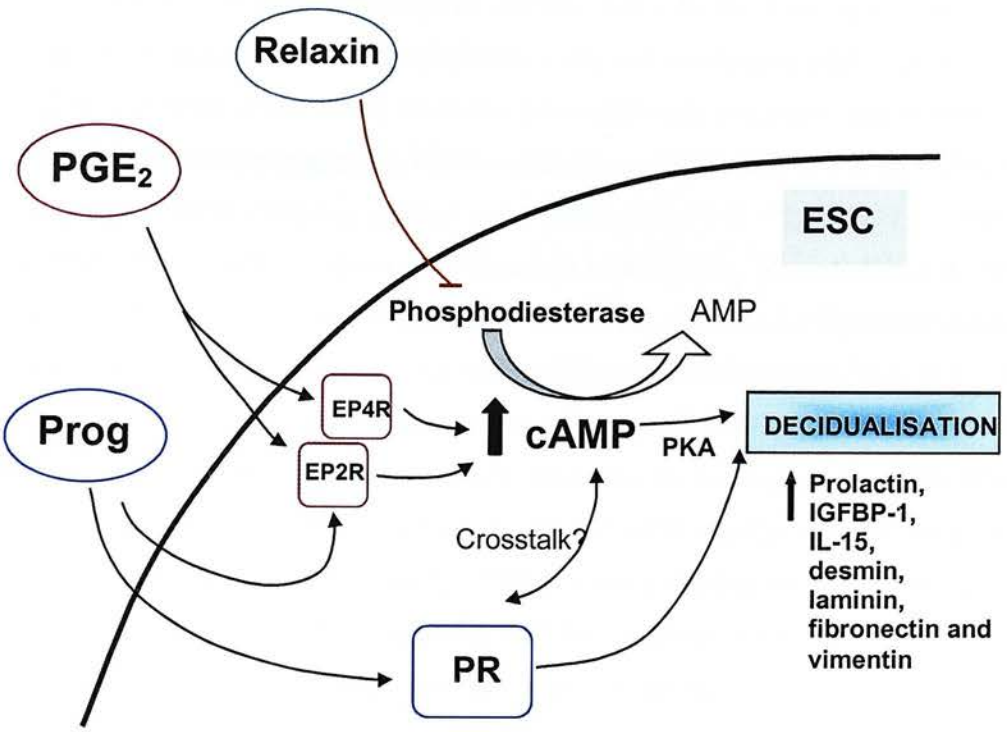


Figure 3.26

A model for the potential mechanisms by which intracellular cAMP, via the PKA signalling pathway, may be playing a central role in ESC decidualisation. This illustrates a possible convergence of the separate pathways of progesterone (**Prog**), **PGE_2** and **relaxin** during transformation of the stroma.

Several studies have utilised gene array technology to analyse changes in gene expression within the human endometrium and many of these are relevant to the time of decidualisation. These studies provide information on a wide range of genes that are being either up- or down-regulated at specific times in the menstrual cycle. Genes thought to be important in the decidualisation reaction are summarised in table 3.7. The regulation of these genes during the luteal phase and early pregnancy has been explored by the different research groups. For example, the up-regulation of IGFBP-1, known to be a product of decidualising stromal cells, between day LH + 2 and LH + 7 has been confirmed by use of this technology (Riesewijk *et al.*, 2003). Phospholipase A_2 (PLA_2) is a rate-limiting step in the PG pathway. The levels of PLA_2 are increased during the implantation window compared with late proliferative endometrium (Kao *et al.*, 2002) and are also raised in first trimester decidua (Chen *et al.*, 2002). In addition, one of the PG synthases, PGD2 synthase, is more abundant in the secretory phase than the proliferative based on a ranking system of the 200 most abundant transcripts in each of the phases (Borthwick *et al.*, 2003). This evidence implies an activation of the PG pathway at a time where decidualisation will be occurring in the stromal compartment. VEGF is an endothelial mitogen and produced in the human endometrium. In mid-secretory phase human endometrium VEGF immuno-staining is observed in the stromal cells and isolated ESCs expressed significantly raised levels of VEGF mRNA in concert with decidualisation in these cells (Sugino *et al.*, 2002). This is in agreement with a study that has shown cAMP-induced decidualisation *in vitro* induces VEGF mRNA expression (Popovici *et al.*, 1999). These gene array-based studies on whole endometrium do not, however, provide information on the specific cellular location of these regulated genes but are an important starting point and an effective approach to determine novel factors involved in key reproductive processes.

Table 3.7

A summary of array-based data recently published concerning genes relevant to decidualisation.

Gene	Report	Reference
IGFBP-1	(i) Increased expression in ESCs decidualised *in vitro* (ii) 2.8-fold up-regulation during the implantation window compared to late proliferative endometrium (iii) Higher gene expression in decidua versus chorionic villi	(i) (Popovici *et al.*, 2000) (ii) (Kao *et al.*, 2002) (iii) (Chen *et al.*, 2002)
Desmin	Higher expression in chorionic villi versus decidua	(Chen *et al.*, 2002)
Vimentin	Rank as the most abundant transcript present is lower in the secretory phase than in the proliferative phase	(Borthwick *et al.*, 2003)
Laminin	(i) Laminin S β3 chain and laminin β1 chain increase in expression during the implantation window compared to late proliferative endometrium (ii) Laminin β3 increases 15-fold between day LH +2 and LH +7	(i) (Kao *et al.*, 2002) (ii) (Riesewijk *et al.*, 2003)
Prostaglandin	(i) 3.6-fold up-regulation of the prostaglandin receptor EP_2 during the implantation window compared to late proliferative endometrium (ii) 18-fold up-regulation of PLA_2 during the implantation window compared to late proliferative endometrium (iii) High level of PLA_2 gene expression detected in first trimester decidua	(i) (Kao *et al.*, 2002) (ii) (Kao *et al.*, 2002) (iii) (Chen *et al.*, 2002)
IL-15	(i) 2.2-fold increase in IL-15 gene expression between the early and mid luteal phases (ii) Increase in IL-15 expression in ESCs decidualised *in vitro* (iii) IL-15, IL-15 precursor and IL-15 receptor α chain precursor genes are up-regulated during the implantation window compared to late proliferative endometrium	(i) (Carson *et al.*, 2002) (ii) (Popovici *et al.*, 2000) (iii) (Kao *et al.*, 2002)
VEGF	(i) Increase in VEGF expression in ESCs decidualised *in vitro* (ii) 3.4-fold decrease in VEGF gene expression during the implantation window compared to late proliferative endometrium	(i) (Popovici *et al.*, 2000) (ii) (Kao *et al.*, 2002)

The results detailed in this chapter demonstrate the up-regulation of IL-15 mRNA expression in concert with increasing prolactin and IGFBP-1 mRNA and protein levels. The required presence of progesterone and cAMP together have the ability to induce both decidualisation and IL-15 mRNA expression effectively. This supports the concept of a link between decidualising ESCs and IL-15 production. It has previously been suggested that IL-15 may be linked with progesterone-induced decidualisation in human ESCs *in vitro* (Okada *et al.*, 2000a). In agreement with these findings analysis using Microarray Technology (refer to table 3.7) has revealed a rise in the IL-15 mRNA in human ESCs treated with decidualising stimuli *in vitro* (Popovici *et al.*, 2000). Raised levels of the IL-15 gene have been documented in the mid-luteal phase (Carson *et al.*, 2002) and in addition the IL-15 precursor and IL-15 receptor α chain precursor genes are up-regulated during the implantation window (Kao *et al.*, 2002). These data provide evidence for both amplification of IL-15 production and also in the actions of this cytokine via an increase in the IL-15 receptor α chain subunit. It is possible that this rise in IL-15 receptor α chain precursor gene is due to the expansion of uterine natural killer cells during this cycle stage and it is thought that these cells may have a role in implantation (King *et al.*, 1990).

The role of PGE_2 in decidualisation has been explored in this chapter. It appears that the presence of progesterone is essential for decidualisation to take place via PGE_2. Alone, neither PGE_2 nor MPA had appreciable effects on decidualisation *in vitro* as determined by prolactin and IGFBP-1 levels. When they were added together they appeared to act in synergy proving to be an effective combination for a decidualisation stimulus. This confirms the findings of a previous study by Frank *et al* 1994 (Frank *et al.*, 1994). Rats treated with indomethacin, an inhibitor of COX, exhibit a reduction in the proportion of pregnant rats and a significant reduction in uterine decidualisation (Sookvanichsilp *et al.*, 2002). COX exists in two isoforms, COX-1 and-2, and can be a rate-limiting step in progression of the PG pathway in addition to phospholipase activity. These experiments have demonstrated further the role of the PGE_2 pathway in the process of decidualisation.

In summary, PGE_2 and the synthetic progestin, MPA, are weak inducers of prolactin and IGFBP-1 expression. However, when added in combination they appear to have synergistic actions. The effects of PGE_2 are likely to be via its ability to raise intracellular cAMP levels since addition of a synthetic cAMP analogue that is resistant to phosphodiesterases in combination with MPA dramatically raises levels of the decidualisation markers. Decidualisation of human ESCs *in vitro* is likely to be an irreversible reaction and therefore one of the reasons that makes menstruation necessary in humans. The process of ESC decidualisation appears to be linked with IL-15 mRNA expression. Those treatments with the ability to induce the greatest rises in prolactin and IGFBP-1 levels also raise IL-15 expression in concert. This relationship between decidualisation of ESCs and IL-15 provides further evidence of a link between the stromal cells and uNK cells.

4. The Prostaglandin Pathway and Decidualisation

4.1 Introduction

Prostaglandin ligand-receptor interactions are key to many reproductive functions such as ovulation, implantation, decidualisation and parturition (Lim *et al.*, 1997). Prostaglandin (PG) production is dependent on a complex intracellular signalling pathway with two major rate-limiting stages: the availability of free substrate, arachidonic acid (AA), via the activity of Phospholipase A_2 and secondly COX activity, the enzyme responsible for catalysing the conversion of AA into the intermediate, PGH_2. COX exists in two isoforms, COX-1, which is expressed constitutively, and the inducible form, COX-2. However, in certain circumstances COX-1 can be induced (Jackson *et al.*, 1993) (Brannon *et al.*, 1994) and COX-2 can be expressed constitutively in the absence of pathology (Walenga *et al.*, 1996). These isoforms are encoded by two separate genes and also differ in their sub-cellular locality and regulation. PGH_2 is converted into the various prostanoids and thromboxanes via specific PG synthases (Smith *et al.*, 1996) (Smith *et al.*, 1996), which can then act on specific receptors to mediate their actions. The prostanoid PGE_2 acts on the EP-receptors: EP_1, EP_2, EP_3 and EP_4 (Narumiya *et al.*, 1999) (Coleman *et al.*, 1994). The EP_2 and EP_4 receptor subtypes exert their effects through raising cAMP (Figure 4.1) (refer to chapter 1.4.1 for further details). The EP_3 receptor also acts via the cAMP pathway and can either result in a rise or reduction in cAMP levels. However, the overall effect is a reduction in cAMP levels (Adam *et al.*, 1994; Sugimoto *et al.*, 2000). The EP_1 receptor acts via the IC Ca^{2+} signalling pathway upon stimulation (Kennedy *et al.*, 1982; Watabe *et al.*, 1993).

Within the human endometrium PGE_2 has been localised to the epithelial, endothelial and stromal compartments in the functional layer (Milne *et al.*, 2001). The role of PGE_2 in female reproductive processes has been analysed via knockout mice for COX-1 and COX-2. These studies have demonstrated the involvement of COX-2 but not COX-1 in implantation and decidualisation (Reese *et al.*, 2001) (Chakraborty *et al.*, 1996) (Langenbach *et al.*, 1995) (Figure 4.2). However, it has been demonstrated that COX-2 may be compensating for COX-1 deficiency during implantation in COX-1 knockouts

(Reese *et al.*, 1999). It has not been possible to study double knockout mice for COX-1 and COX-2 since they fail to live long postnatally (Loftin *et al.*, 2001).

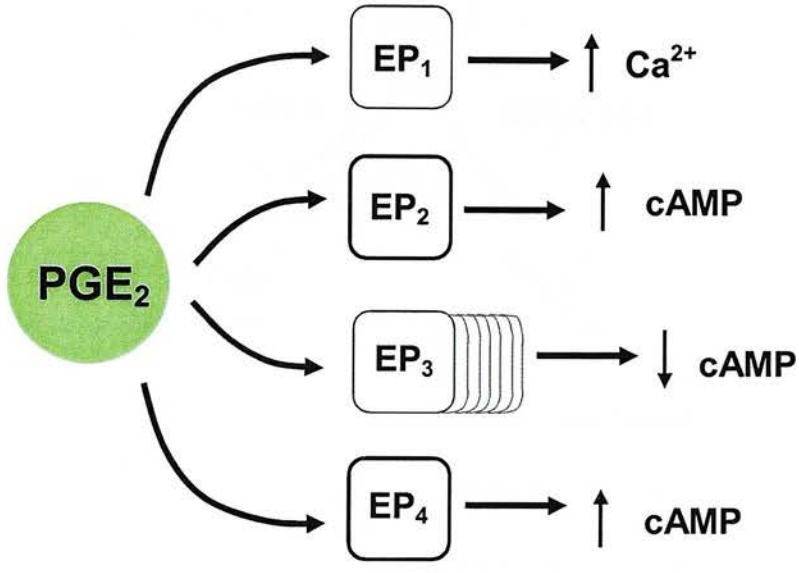


Figure 4.1

Illustration of PGE_2 acting via the EP_2 and EP_4 receptors to raise intracellular cAMP levels. The EP_3 receptor exists as multiple splice variants, and acts via G_i proteins to have the predominant effect of decreasing cAMP levels.

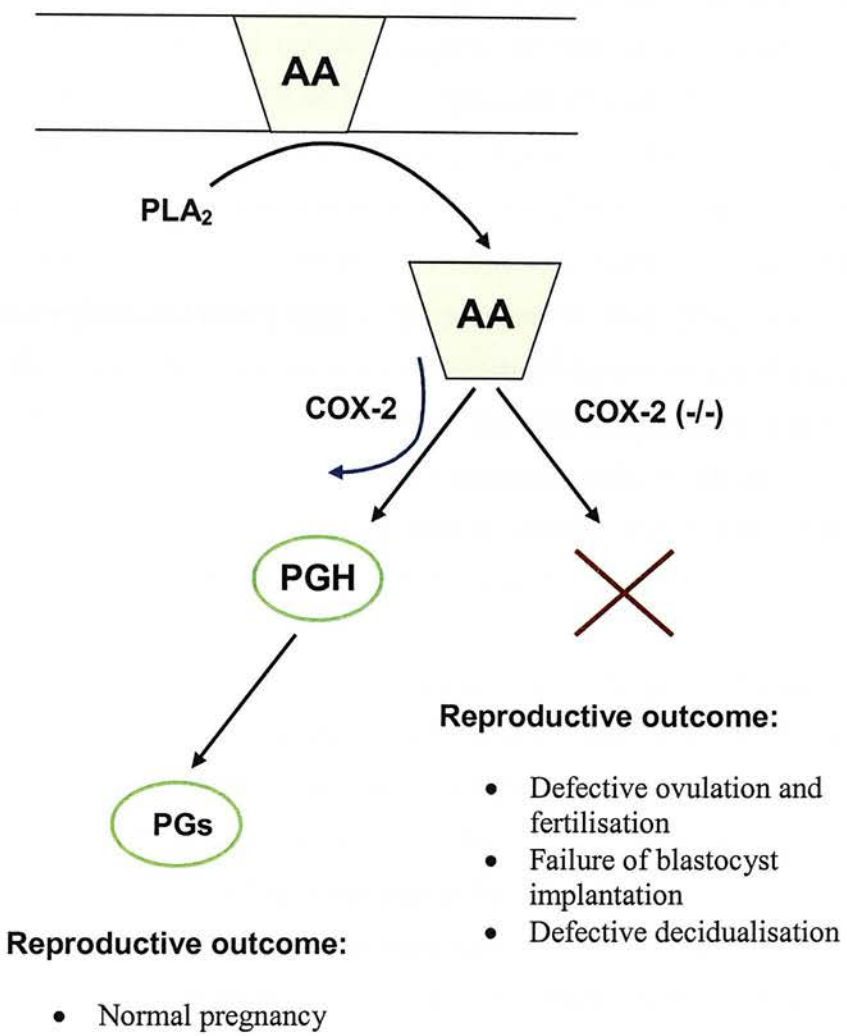


Figure 4.2

The effect of the PG pathway progression on reproductive outcomes in wild type mice versus COX-2 (-/-) mice illustrating the importance of PGs in female fertility. Phospholipase A_2 (PLA_2) is the enzyme responsible for liberating membrane-bound arachidonic acid (AA). AA is then converted into the intermediate, PGH, via COX-2 enzyme under normal circumstances. Thus, in COX-2 (-/-) mice the PG pathway is ablated, preventing the formation of any prostanoids.

A link between the actions of progesterone and PGs in the uterus has been demonstrated via studies on the effects of the anti-progestin, RU486, in the human endometrium (Marions *et al.*, 1999). Levels of COX-1 declined whereas COX-2 immunostaining persisted further illustrating the differences in regulation between these two enzymes. Endometrial PGs were measured at different times of the menstrual cycle and it was observed that the $PGF_{2\alpha}$: PGE_2 ratio increase from the proliferative phase, peaking in the mid-secretory phase and falling again at menstruation (Downie, 1974). $PGF_{2\alpha}$ and PGE_2 concentrations were measured in human endometrial biopsies by gas chromatography-mass spectrometry (Maathuis *et al.*, 1978). The PGF concentration was found to be greatest in the mid-secretory phase and no variation in PGE_2 levels was observed. An increase in PGF production is also apparent in cultured human ESCs and glandular epithelial cells following RU486 treatment (Kelly *et al.*, 1986).

The involvement of PGE_2 in the decidual transformation of the endometrial stroma has been previously suggested following *in vitro* culture experiments using human and rat ESCs (Frank *et al.*, 1994; Kennedy *et al.*, 1982; Peleg, 1983). They demonstrated that the combination of PGE_2 and progesterone is able to induce decidualisation in these cell cultures. The exact intracellular mechanisms by which this is occurring have not yet been analysed. COX-2 (-/-) mice have been shown to exhibit defective decidualisation (Lim *et al.*, 1997) supporting evidence for the involvement of PGE_2 in stromal differentiation. In studies on cultured human ESCs treated with hCG to induce differentiation, an increase in COX-2 gene expression was observed via raised transcript stability (Han *et al.*, 1996) providing further evidence for a role of PGs in decidualisation.

This chapter examines changes occurring in components of the PG pathway in decidualised versus non-decidualised human ESCs *in vitro*. In addition, the effect of decidualising pre-treatments on the release of PGE_2 and $PGF_{2\alpha}$ protein from human ESCs *in vitro* has been explored.

4.2 Methods

4.2.1 Human Uterine Tissue Collection

Endometrial (n = 14) biopsies (7 proliferative, 6 early secretory and 1 mid secretory) were collected and processed as described in section 2.1. All samples were processed to separate the ESCs from the glands, as described in section 2.2. The cells were grown for a period of two weeks prior to commencing experiments in the presence of MPA and bFGF.

4.2.2 Human Primary Cell Culture

4.2.2a *in vitro* Decidualisation of Primary ESCs

ESCs were seeded in 12-well plates at a concentration of $1.4x10^5$ cells/ml and allowed to adhere overnight. The following treatment regimes were followed as detailed in tables 4.1 and 4.2. The experiments were designed to stimulate *in vitro* decidualisation of ESCs (refer to chapter 3) and allow the analysis of alterations in the PG signalling pathway via Q RT-PCR.

Table 4.1

Experiment 1: Decidualisation of ESCs (n = 4)		
Treatment	**Concentration**	**Incubation time**
Control	N/A	4, 8 and 12 days
MPA	10^{-6} M	
PGE_2	10^{-6} M	
PGE_2 + MPA	10^{-6} M + 10^{-6} M	
8-Bromo cAMP	250μM	
8-Bromo cAMP + MPA	250μM + 10^{-6} M	

Table 4.2

Experiment 2: Decidualisation of ESCs and treatment removal (n = 5)		
Treatment	**Concentration**	**Incubation time**
Control	N/A	5 days, 5 days followed by 5 days with treatment withdrawal, 10 days continuous treatment
PGE_2 + MPA	10^{-6} M + 10^{-6} M	
8-Bromo cAMP + MPA	250μM + 10^{-6} M	

Decidualisation in these treated cell cultures was confirmed by prolactin and IGFBP-1 ELISA and Q RT-PCR (refer to section 3.3).

4.2.2b Effect of Pre-treating ESCs with Decidualising Stimuli on PGE_2 and $PGF_{2\alpha}$ Release

ESCs were seeded in 12-well plates at a concentration of $1.4x10^5$ cells/ml and allowed to adhere overnight. The following treatment regime adhered to is detailed in table 4.3. The experimental design was to establish the effects of decidualising stimuli on the secretion of PGE_2 and $PGF_{2\alpha}$ by human ESCs *in vitro*.

Table 4.3

Experiment 3: Decidualisation Pre-treatment of ESCs (n = 5)		
Pre-treatment	**Concentration**	**Incubation time**
Control	N/A	4 days pre-treatment followed by 2 days with complete RPMI alone.
MPA	10^{-6} M	
PGE_2	10^{-6} M	
PGE_2 + MPA	10^{-6} M + 10^{-6} M	
8-Bromo cAMP	250μM	
8-Bromo cAMP + MPA	250μM + 10^{-6} M	

4.2.3 RNA Extraction and Q RT-PCR

RNA was extracted from the ESC cultures at the termination of the experiments 1 and 2 and cDNA prepared as detailed in sections 2.4 and 2.5. Messenger RNA expression of the following components of the PG cascade was measured by Q RT-PCR: COX-1, COX-2, Phosphodiesterase3a (PDE3a), Phosphodiesterase4b (PDE4b), Prostaglandin transporter protein (PGTP) and EP_2 and EP_4 receptors.

4.2.4 PGE_2 and $PGF_{2\alpha}$ ELISAs

Media was collected at the termination of experiment 3 and processed for ELISAs. Refer to Methods chapter, sections 2.6.2 for a description of a Competition ELISA.

4.2.4a $PGF_{2\alpha}$ ELISA

The general method description for an ELISA is detailed in section 2.6. For measurement of $PGF_{2\alpha}$ by ELISA a buffer with no Tween added is used. The standard curve range is 5120pg/ml to 10pg/ml with 1:2 dilutions to give 10 standards in total. The Quality control is set at 200pg/ml in a minimum of 4 wells per plate. All wells received 50µl AS, 50µl Link and 100µl of either a standard or a sample. The link increases the sensitivity of the assay via proline coupling to a PG, making it more antigenic. This enables the free PG in the sample to displace the biotin-labelled link for AS binding sites (Kelly *et al.*, 1989). The well to show non-specific binding (NSB) receives 50µl Link and 150µl of buffer and the B_0 well receives 50µl AS, 50µl Link and 100µl of buffer. The plate was incubated at 4°C overnight. The following day plates are washed four times, once for 30 seconds on a plate shaker. The streptavidin was diluted in buffer (no Tween) to a concentration of 0.125U/ml and 100µl pipetted into all wells. Plates were placed on a plate shaker at room temperature for 20 minutes before being washed as before. The substrate (as in the Two-site sandwich ELISA) was added to the wells, 200µl per well. Plates were left for 20m before being quenched with 2N

Sulphuric Acid and read on a plate reader. A method file was designed in Assay Zap (section 2.6) and used to analyse results. Intra-assay variation was calculated from 10 replicates across the same plate and inter-assay variation was calculated from across a minimum of 6 different assay plates and these were calculated to be 7.8% and 15.0% respectively.

4.2.4b PGE_2 ELISA

For details of the procedure see Competition ELISA and $PGF_{2\alpha}$ ELISA protocol details above. Exceptions are that all samples were MOXed upon collection 1:1 with Methyl oximating (MoxB) solution in order to stabilise to PGE_2 and prevent conversion to the PGA form. Standards for the plate were made up in a 25% MOX buffer (MoxB diluted in B+T). The top standard is 1048pg/ml and the bottom standard is 2.8pg/ml (10 standards in total). A Link and Anti-sera specific for PGE_2 were used. Intra-assay variation was calculated to be 5.2% and inter-assay variation was 18.3%.

4.2.5 Statistical Analysis

Q RT-PCR and ELISA data were analysed by the methods described previously in section 2.8.

4.3 Results

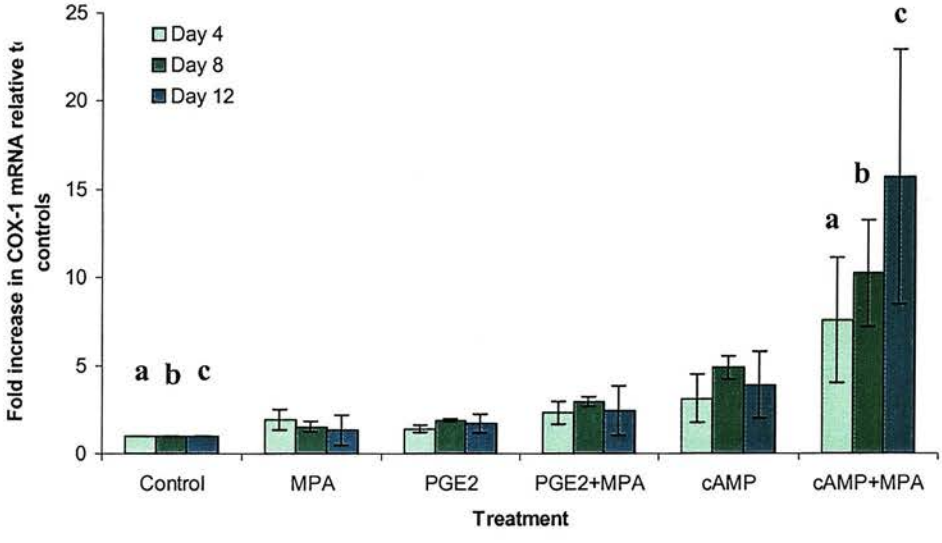
4.3.1a COX-1 mRNA Expression in Decidualising ESCs

Treatment of ESC cultures with PGE_2 plus MPA stimulated an increase in COX-1 mRNA levels of between 2- and 3-fold relative to controls (figure 4.3). The expression of COX-1 mRNA was increased between 3- and 4-fold following 8-Bromo cAMP treatment on days 4, 8 and 12. The combination of 8-Bromo cAMP plus MPA triggered the greatest rises in COX-1 expression, with 8-, 10- and 16-fold increases observed on days 4, 8 and 12 respectively ($p < 0.010$; $p < 0.0012$; $p < 0.0032$).

Figure 4.3

The effects of decidualising stimuli on the expression of COX-1 mRNA in human ESCs after 4, 8 and 12 days of treatment. Same letters denote significant difference **a** $p < 0.010$; **b** $p < 0.0012$ and **c** $p < 0.0032$.

Figure 4.3


Day 4
Day 8
Day 12
Fold increase in COX-1 mRNA relative t
controls
25
20
15
10
5
0
a b c
a
b
c
Control
MPA
PGE2
PGE2+MPA
cAMP
cAMP+MPA
Treatment

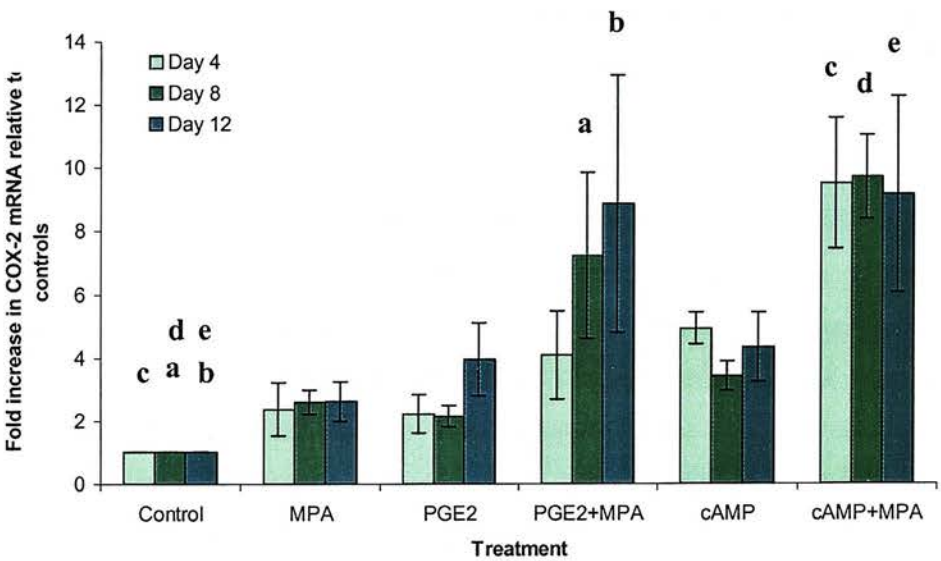
4.3.1b COX-2 mRNA Expression in Decidualising ESCs

The expression of COX-2 mRNA increased 2.5-fold with MPA treatment compared with controls (figure 4.4). PGE_2 stimulated a 2-fold increase in COX-2 levels on days 4 and 8 and this increased to a 4-fold increase on day 12. The combination of PGE_2 plus MPA resulted in 4- (NS $p < 0.07$), 7- ($p < 0.0022$) and 9-fold ($p < 0.021$) rises in COX-2 mRNA on days 4, 8 and 12 respectively. Alone, 8-Bromo cAMP stimulated a 4-fold rise in levels but when added in combination with MPA, stimulated a 9-fold elevation across all time-points ($p < 0.0001$; $p < 0.0001$; $p < 0.017$).

Figure 4.4

The effects of decidualising stimuli on the expression of COX-2 mRNA in human ESCs after 4, 8 and 12 days of treatment. Same letters denote significant difference. **a** $p < 0.0022$; **b** $p < 0.021$; **c** $p < 0.0001$; **d** $p < 0.0001$ and **e** $p < 0.017$.

Figure 4.4


Day 4
Day 8
Day 12
Fold increase in COX-2 mRNA relative t
controls
14
12
10
8
6
4
2
0
c a b
d e
a
b
c d
e
Control
MPA
PGE2
PGE2+MPA
cAMP
cAMP+MPA
Treatment

4.3.1c COX-1 mRNA Expression in Decidualised ESCs and Pre-decidualised ESCs after Decidualising Treatment Removal

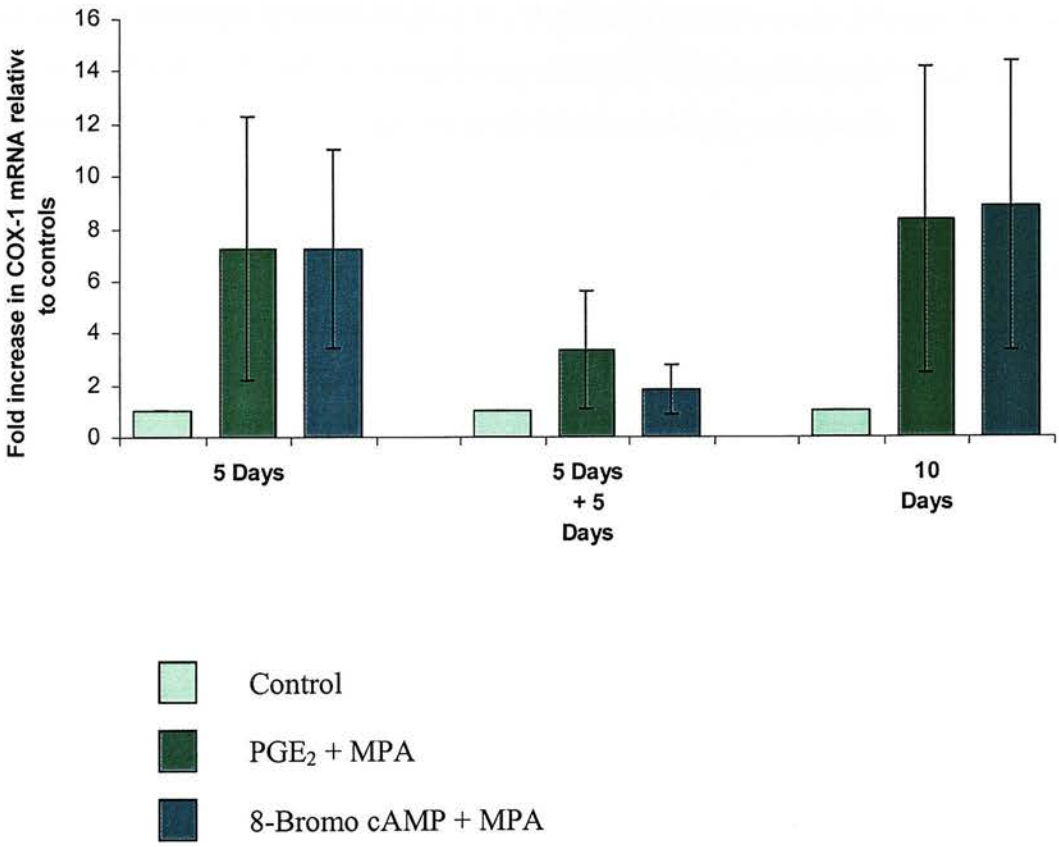
Figure 4.5 shows the expression of COX-1 mRNA in decidualising ESCs after 5 and 10 days of treatment and also after 5 days of continuous treatment followed by 5 days of no treatment. A 7-fold increase relative to controls in COX-1 mRNA was stimulated by both cAMP plus MPA and by PGE_2 plus MPA treatments after 5 days of continuous treatment. However, this result failed to reach significance due to one ESC culture sample expressing a much higher level of COX-1 than the other 4. If this result is omitted the results are significant: 8-Bromo cAMP plus MPA $p < 0.001$ and PGE_2 plus MPA $p < 0.05$. The same trends were maintained after 10 days of continuous treatment although were not statistically significant. However, when the decidualising stimuli were removed for 5 days following 5 days of pre-treatment levels of COX-1 mRNA declined to those similar to controls.

The statistics used to assign significance was the parametric ANOVA. This test assumes a normal distribution, as was expected from our samples, and is more powerful than non-paramentric tests. However, because one of the samples was expressing much greater levels of COX-1 mRNA compared with its own control than the other samples were, this made the results insignificant even though the same trend was occurring in all samples. A Kruskal-Wallis Test (non-parametric ANOVA) was then performed on this data and Dunn's Multiple Comparisons test used to assign significance. The medians in each group were compared with each other. This test showed there to be a significant difference between the control group and the group treated with cAMP plus MPA on treatment days 5 and 10 ($p < 0.01$ and 0.05 respectively). With the remaining data that has been assessed throughout this study no other out-lying results have been present to affect the possibility of significance. Therefore, because the remaing data appears to represent a normal distribution the parametric ANOVA test was utilised.

Figure 4.5

The effects on COX-1 mRNA expression of ESCs treated with decidualising stimuli for either 5 or 10 days continuously or for 5 days followed by removal of treatment for a further 5 day period.

Figure 4.5


Fold increase in COX-1 mRNA relative to controls
16
14
12
10
8
6
4
2
0
5 Days
5 Days + 5 Days
10 Days
Control
PGE2 + MPA
8-Bromo cAMP + MPA

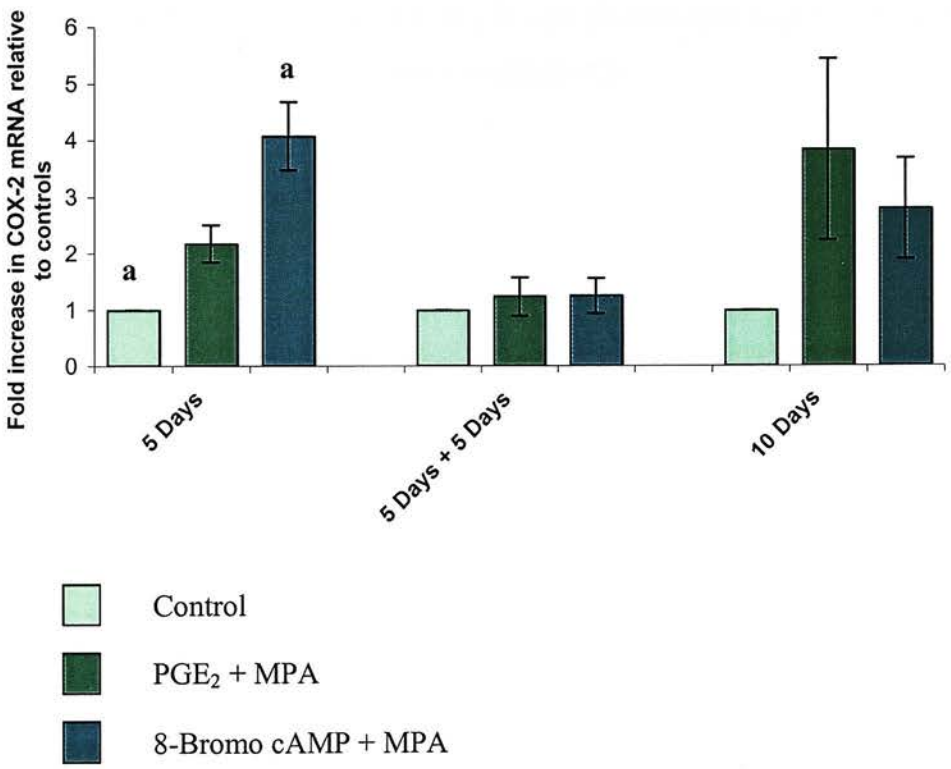
4.3.1d COX-2 mRNA Expression in Decidualised ESCs and Pre-decidualised ESCs after Decidualising Treatment Removal

Following 5 and 10 days of continuous treatment with PGE_2 plus MPA, 2- ($p < 0.055$ NS) and 4-fold increases in COX-2 mRNA levels relative to controls were observed respectively (figure 4.6). Treatment of ESCs with 8-Bromo cAMP plus MPA stimulated a 4-fold rise in COX-2 mRNA expression after 5 days ($p < 0.0002$) and after 10 days of continuous treatment this had dropped to a 3-fold increase relative to controls. In those ESCs subjected to 5 days of decidualising treatment with a subsequent 5 days in the absence of treatments, COX-2 mRNA levels had returned to control levels.

Figure 4.6

The effects on COX-2 mRNA expression of ESCs treated with decidualising stimuli for either 5 or 10 days continuously or for 5 days followed by removal of treatment for a further 5 day period. **a** $p < 0.0002$.

Figure 4.6


Fold increase in COX-2 mRNA relative to controls
6
5
4
3
2
1
0
a
a
5 Days
5 Days + 5 Days
10 Days
Control
PGE2 + MPA
8-Bromo cAMP + MPA

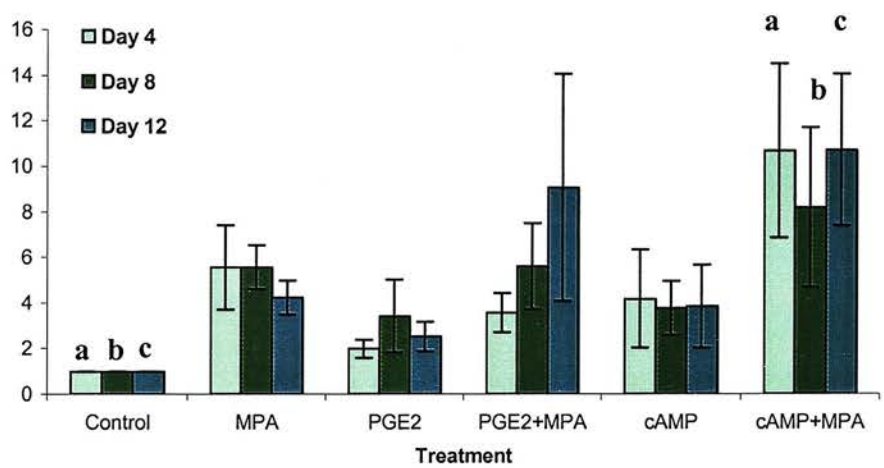
4.3.1e EP_2 Receptor mRNA Expression in Decidualising ESCs

The expression of EP_2 receptor mRNA rose 6-fold compared with equivalent controls after 4 days of MPA treatment (figure 4.7). PGE_2 treatment stimulated 2- and 3-fold increases in expression levels after 4 and 8 days respectively. Treating cells with the combination of MPA and PGE_2 did not stimulate any further increase in EP_2 receptor expression. ESCs treated with cAMP alone exhibited a 4-fold elevation in relative expression after 4, 8 and 12 days of treatment. Where cAMP was added alongside MPA the expression levels were elevated by 10-, 8- and 10-fold after 4 ($p < 0.003$), 8 ($p < 0.013$) and 12 days ($p < 0.013$) of treatment respectively.

Figure 4.7

The effects of decidualising stimuli on the expression of the EP_2 receptor in human ESCs after 4, 8 and 12 days of treatment. Same letters denote significant difference. **a** $p < 0.003$; **b** $p < 0.013$ and **c** $p < 0.013$.

Figure 4.7


16
14
12
10
8
6
4
2
0
Day 4
Day 8
Day 12
a b c
a
b
c
Control
MPA
PGE2
PGE2+MPA
cAMP
cAMP+MPA
Treatment

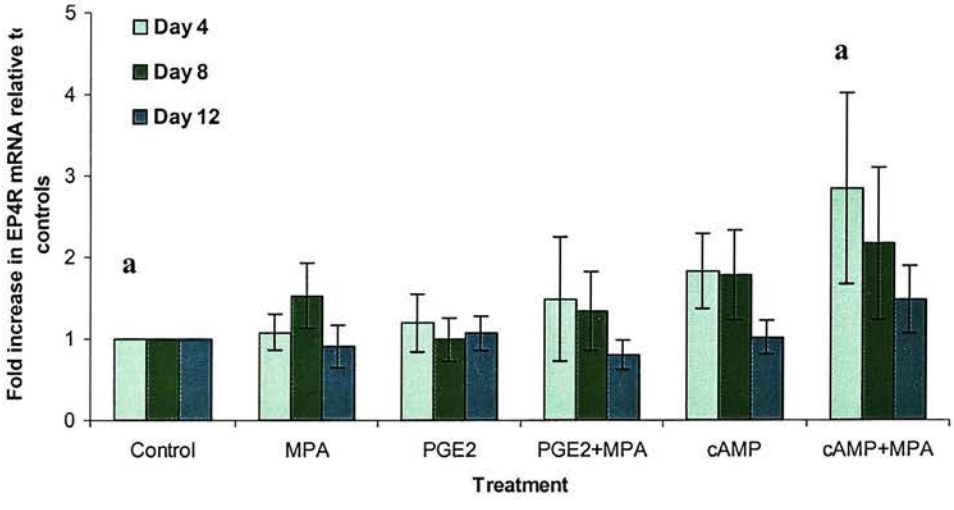
4.3.1f EP_4 Receptor mRNA Expression in Decidualising ESCs

The expression of the EP_4 receptor mRNA was relatively similar to controls across the treatment regime added to ESC cultures and time points with the exception of treatment of cells for 4 days with 8 Bromo-cAMP plus MPA ($p < 0.05$) (figure 4.8). This significant rise in EP_4 receptor appears to be temporal with a reduction on day 8 and further on day 12 with relative expression becoming more comparable with control levels.

Figure 4.8

The effects of decidualising stimuli on the expression of the EP_4 receptor in human ESCs after 4, 8 and 12 days of treatment. Same letters denote significant difference. **a** $p < 0.05$.

Figure 4.8


Day 4
Day 8
Day 12
Fold increase in EP4R mRNA relative to controls
5
4
3
2
1
0
a
a
Control
MPA
PGE2
PGE2+MPA
cAMP
cAMP+MPA
Treatment

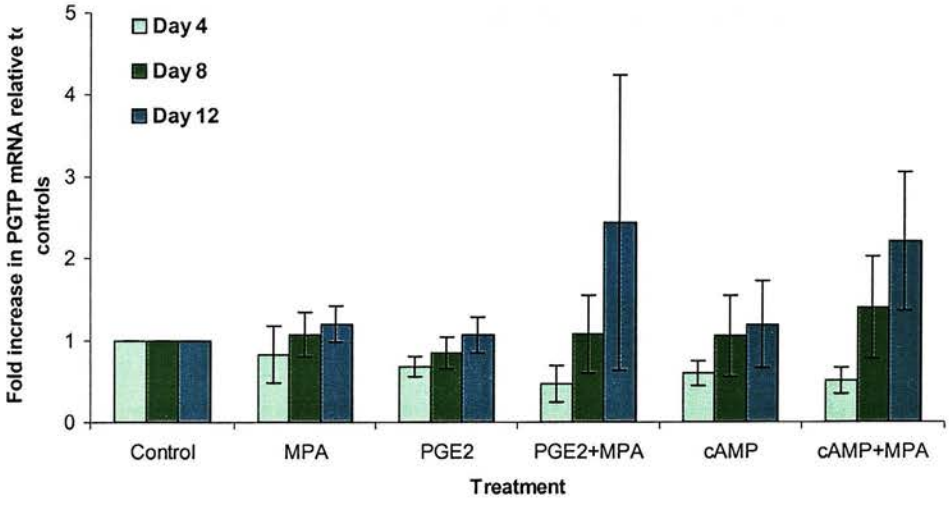
4.3.1g Prostaglandin Transporter Protein (PGTP) mRNA Expression in Decidualising ESCs

Figure 4.9 illustrates the relative mRNA expression of the PGTP mRNA in treated ESCs. Levels of expression were similar to controls across all treatment groups at all time points with the exception of day 12. A 2-fold increase in PGTP mRNA relative to controls was observed after PGE_2 plus MPA or 8 Bromo-cAMP plus MPA treatment but this was not a significant rise.

Figure 4.9

The effects of decidualising stimuli on the mRNA expression of the PGTP in human ESCs after 4, 8 and 12 days of treatment.

Figure 4.9


Day 4
Day 8
Day 12
Fold increase in PGTP mRNA relative to controls
5
4
3
2
1
0
Control
MPA
PGE2
PGE2+MPA
cAMP
cAMP+MPA
Treatment

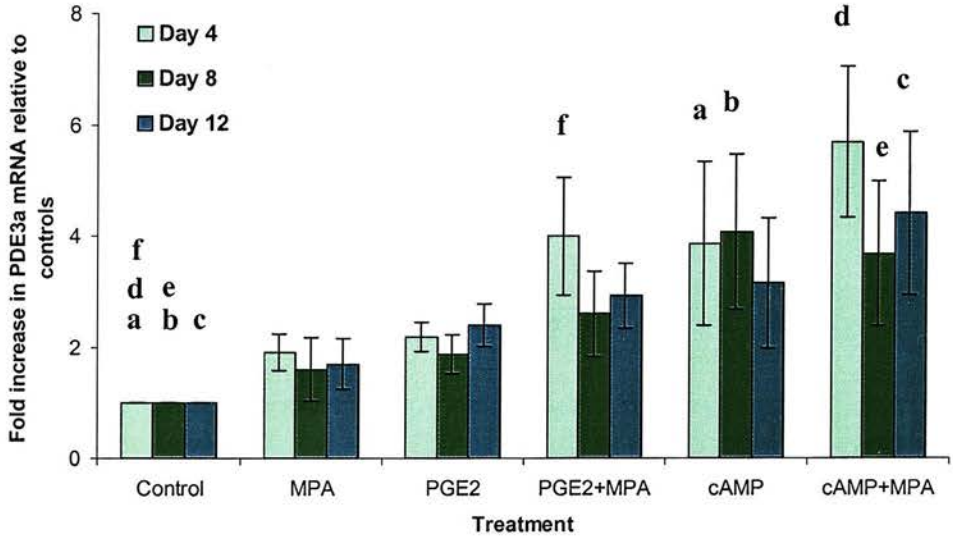
4.3.1h Phosphodiesterase 3a (PDE3a) mRNA Expression in Decidualising ESCs

Figure 4.10 represents the expression of PDE3a mRNA levels relative to controls following 4, 8 or 12 days of treatment. ESCs treated with PGE_2 or MPA exhibited a 2-fold increase in mRNA expression at all time points. Addition of PGE_2 plus MPA had maximal effects by day 4 of treatment with a 4-fold rise in expression levels ($p < 0.038$). On days 4 and 8 those ESCs treated with 8-Bromo cAMP displayed a 4-fold increase in expression ($p < 0.045$ and $p < 0.021$ respectively) with a small decline to a 3-fold rise after 12 days of continuous treatment. ESCs treated with 8-Bromo cAMP plus MPA for 4 days expressed a 5-fold increase in PDE3a mRNA ($p < 0.003$) and cultures treated for 8 or 12 days were expressing a 4-fold increase relative to controls ($p < 0.04$ and $p < 0.01$ respectively).

Figure 4.10

The effects of decidualising stimuli on the mRNA expression of the PDE3a in human ESCs after 4, 8 and 12 days of treatment. Same letters denote significant difference. **a** $p < 0.045$; **b** $p < 0.021$; **c** $p < 0.01$; **d** $p < 0.003$; **e** $p < 0.04$ and **f** $p < 0.038$.

Figure 4.10


Day 4
Day 8
Day 12
Fold increase in PDE3a mRNA relative to controls
0
2
4
6
8
f
d e
a b c
f
a b
d
e
c
Control
MPA
PGE2
PGE2+MPA
cAMP
cAMP+MPA
Treatment

4.3.1i Phosphodiesterase 4b (PDE4b) mRNA Expression in Decidualising ESCs

ESCs treated with PGE_2 or MPA expressed similar levels of PDE4b mRNA levels to controls although a modest 2-fold increase after 12 days was observed in the PGE_2 or PGE_2 plus MPA treated cultures (Figure 4.11). Treatment of ESCs with 8-Bromo cAMP alone stimulated an approximate 3-fold elevation at all time points and this was significant on day 4 ($p < 0.0003$). These results were comparable to the 8-Bromo cAMP plus MPA treatment values which were significantly different to controls on day 4 ($p < 0.003$), day 8 ($p < 0.044$) and day 12 ($p < 0.007$).

Figure 4.11

The effects of decidualising stimuli on the mRNA expression of the PDE4b in human ESCs after 4, 8 and 12 days of treatment. Same letters denote significant difference. **a** $p < 0.0003$; **b** $p < 0.003$; **c** $p < 0.044$ and **d** $p < 0.007$.

Figure 4.11

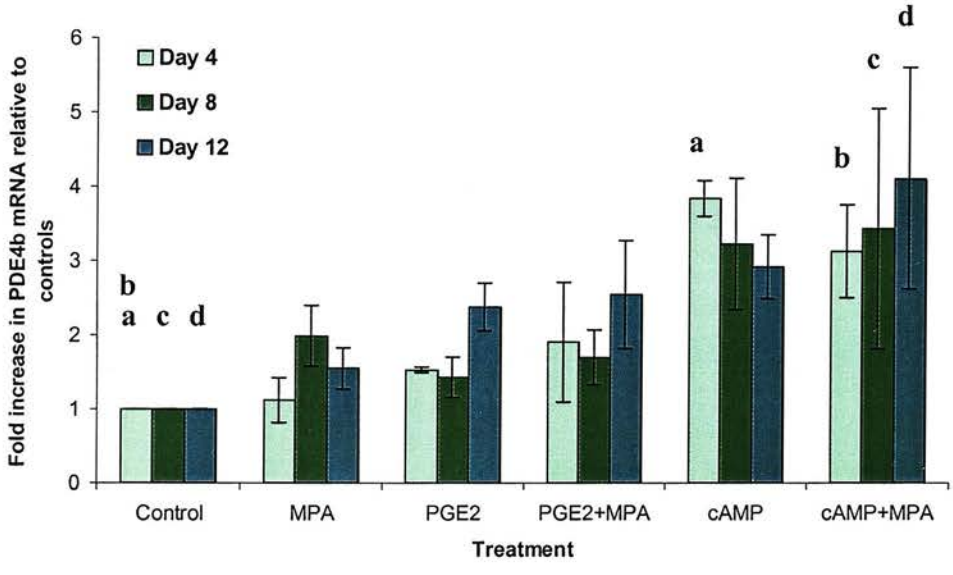

Fold increase in PDE4b mRNA relative to controls
Day 4
Day 8
Day 12
0
1
2
3
4
5
6
b
a c d
a
b
c
d
Control
MPA
PGE2
PGE2+MPA
cAMP
cAMP+MPA
Treatment

Figure 4.12 Release of PGE_2 by ESCs pre-treated with decidualising stimuli for 5 days

The decidualising pre-treatments had no effect on the secretion of endogenous PGE_2 by ESCs. However, PGE_2 treatment alone stimulated a significant rise in PGE_2 secretion ($p < 0.032$). Same letters denote significant difference. **a** $p < 0.032$. The lower detection limit of the ELISA was 20pg/ml.

Figure 4.12

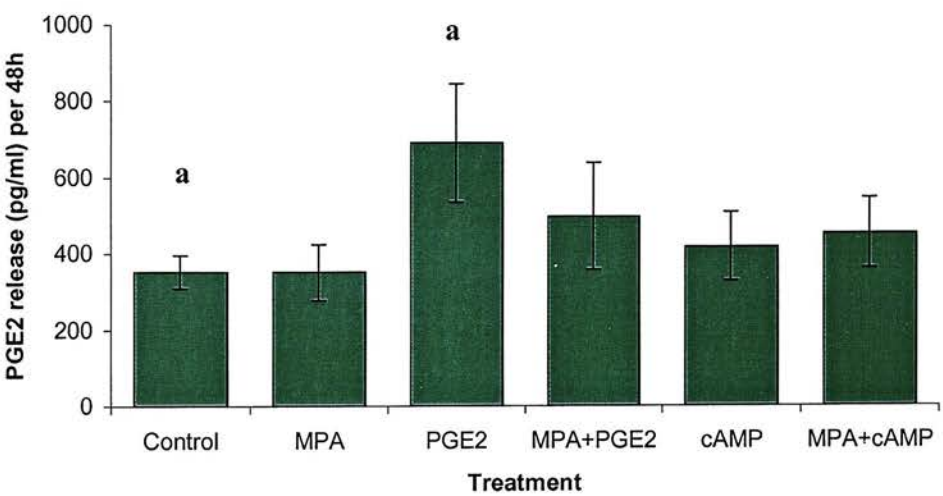

1000
800
600
400
200
0
PGE2 release (pg/ml) per 48h
a
a
Control
MPA
PGE2
MPA+PGE2
cAMP
MPA+cAMP
Treatment

Figure 4.13 Release of $PGF_{2\alpha}$ by ESCs pre-treated with decidualising stimuli for 5 days

ESCs treated with either PGE_2 or 8-Bromo cAMP stimulated an increase in the level of $PGF_{2\alpha}$ protein secretion. Addition of MPA had no additional effect on $PGF_{2\alpha}$ protein secretion and when added alone levels of secretion were comparable to control values. Same letters denote significant difference. **a** $p < 0.0003$; **b** $p < 0.0003$; **c** $p < 0.027$ and **d** $p < 0.0007$. The lower detection limit of the ELISA was 10pg/ml.

Figure 4.13

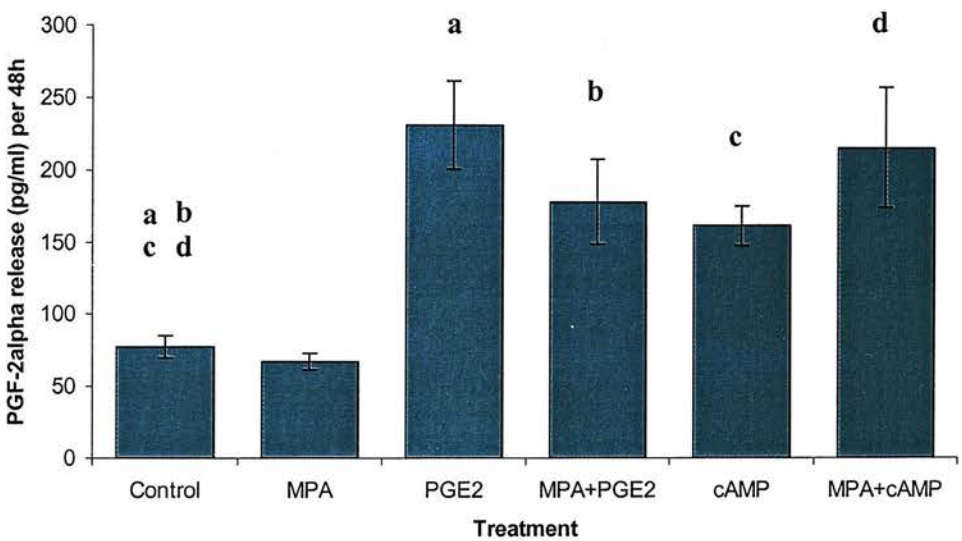

PGF-2alpha release (pg/ml) per 48h
300
250
200
150
100
50
0
a b
c d
a
b
c
d
Control
MPA
PGE2
MPA+PGE2
cAMP
MPA+cAMP
Treatment

Table 4.5 Summary of results for experiment 1 (day 4)

The values in the table represent fold increases in mRNA expression relative to controls with significant differences ($p <$) inserted in brackets where appropriate.

Treatment	COX-1 mRNA	COX-2 mRNA	EP_2R mRNA	EP_4R mRNA	PGTP mRNA	PDE3a mRNA	PDE4b mRNA
Control	1	1	1	1	1	1	1
MPA	1.9	2.4	5.6	1.1	0.8	1.9	1.1
PGE_2	1.4	2.2	2	1.2	0.7	2.2	1.5
PGE_2 + MPA	2.3	4.1	3.6	1.5	0.5	4 (0.038)	1.9
8-Bromo cAMP	3	5	4.2	1.8	0.6	3.9 (0.045)	3.9 (0.0003)
8-Bromo cAMP + MPA	7.6 (0.01)	9.6 (0.0001)	10.8 (0.003)	2.9 (0.05)	0.5	5.7 (0.003)	3.2 (0.003)

Table 4.6 Summary of results for experiment 1 (day 8)

Treatment	COX-1 mRNA	COX-2 mRNA	EP_2R mRNA	EP_4R mRNA	PGTP mRNA	PDE3a mRNA	PDE4b mRNA
Control	1	1	1	1	1	1	1
MPA	1.5	2.6	5.6	1.5	1.1	1.6	2
PGE_2	1.9	2.2	3.4	1	0.9	1.9	1.4
PGE_2 + MPA	3	7.3 (0.0001)	5.6	1.4	1.1	2.6	1.7
8-Bromo cAMP	4.9	3.4	3.8	1.8	1.1	4.1 (0.021)	3.3
8-Bromo cAMP + MPA	10.3 (0.0012)	9.8 (0.0022)	8.3 (0.013)	2.2	1.4	3.7 (0.04)	3.5 (0.044)

Table 4.7 Summary of results for experiment 1 (day 12)

Treatment	COX-1 mRNA	COX-2 mRNA	EP_2R mRNA	EP_4R mRNA	PGTP mRNA	PDE3a mRNA	PDE4b mRNA
Control	1	1	1	1	1	1	1
MPA	1.3	2.6	4.3	0.9	1.2	1.7	1.6
PGE_2	1.7	4	2.6	1.1	1.1	2.4	2.4
PGE_2 + MPA	2.5	8.9 (0.017)	9.1	0.8	2.5	2.9	2.6
8-Bromo cAMP	3.9	4.4	3.9	1	1.2	3.2	2.9
8-Bromo cAMP + MPA	15.8 (0.003)	9.2 (0.021)	10.8 (0.013)	1.5	2.2	4.4 (0.01)	4.1 (0.002)

4.4 Discussion

Decidualisation of the human uterine stroma is likely to be a multi-factorial process. PGE_2 has been implicated as one of these factors contributing markedly to decidualisation of human ESCs *in vitro* (Frank *et al.*, 1994). PGs are able to raise intracellular cAMP levels. This chapter has analysed some of the individual components of the PG pathway in an attempt to assess the effects of decidualisation on it and ultimately on PG production. The results for this chapter are summarised in figures 4.14 and 4.15.

An increase in both COX-1 and COX-2 mRNA expression was observed after 5 and 10 days with continuous decidualising treatment. This effect was diminished upon removal of decidual stimuli illustrating the necessity of sustained stimuli for the rise in the COX enzymes. COX-2 has been implicated in mice decidualisation via studies on COX-2 knockouts where they exhibited defective decidualisation (Lim *et al.*, 1997; Reese *et al.*, 2001) and suggests at some level a link with decidualisation and COX-2. Historically, COX-1 has been considered to be constitutively expressed although emerging evidence shows that it is inducible. In experiments using an embryo lung fibroblast cell line, COX-1 was demonstrated to be regulated by both IL-1β and TGF-β (Jackson *et al.*, 1993). This potential for COX-1 expression to be regulated is in agreement with the results in this chapter. The second rate-limiting step at this point in the pathway is the action of PLA_2, the enzyme responsible for liberating free AA. If AA levels are limited then it is reasonable to expect that the actions of COX-1 and –2 have a threshold effect and therefore the conversion of AA to PGH_2 is limited. This is one of the self-regulatory steps within the PG pathway. The next major stage in the PG pathway is the conversion of PGH_2 to the various prostanoids and thromboxanes via specific PG synthases. PGE synthases (PGES) catalyse the conversion of PDH_2 to PGE_2 and three forms of this enzyme have been identified: cytosolic PGES (Tanioka *et al.*, 2000) and microsomal PGES-1 and –2 (mPGES-1 and –2) (Jakobsson *et al.*, 1999; Murakami *et al.*, 2000; Mancini *et al.*, 2001; Tanikawa *et al.*, 2002). A correlation between COX-2 and PGE synthase expression in bovine endometrial cell cultures has been proposed (Parent *et al.*,

2002) and experiments using a human colorectal adenocarcinoma cell lines showed that abnormally high expression of mPGES-1 and COX-2 together are able to contribute to tumourigenesis (Kamei *et al.*, 2003). As discussed in Murakami *et al* 2003 (Murakami *et al.*, 2003), mPGES preferentially couples to COX-2 over COX-1 and cPGES is able to couple COX-1. If this is the situation in the human endometrium it adds further complexity to control of the PG pathway with a feedback scenario occurring between these two stages.

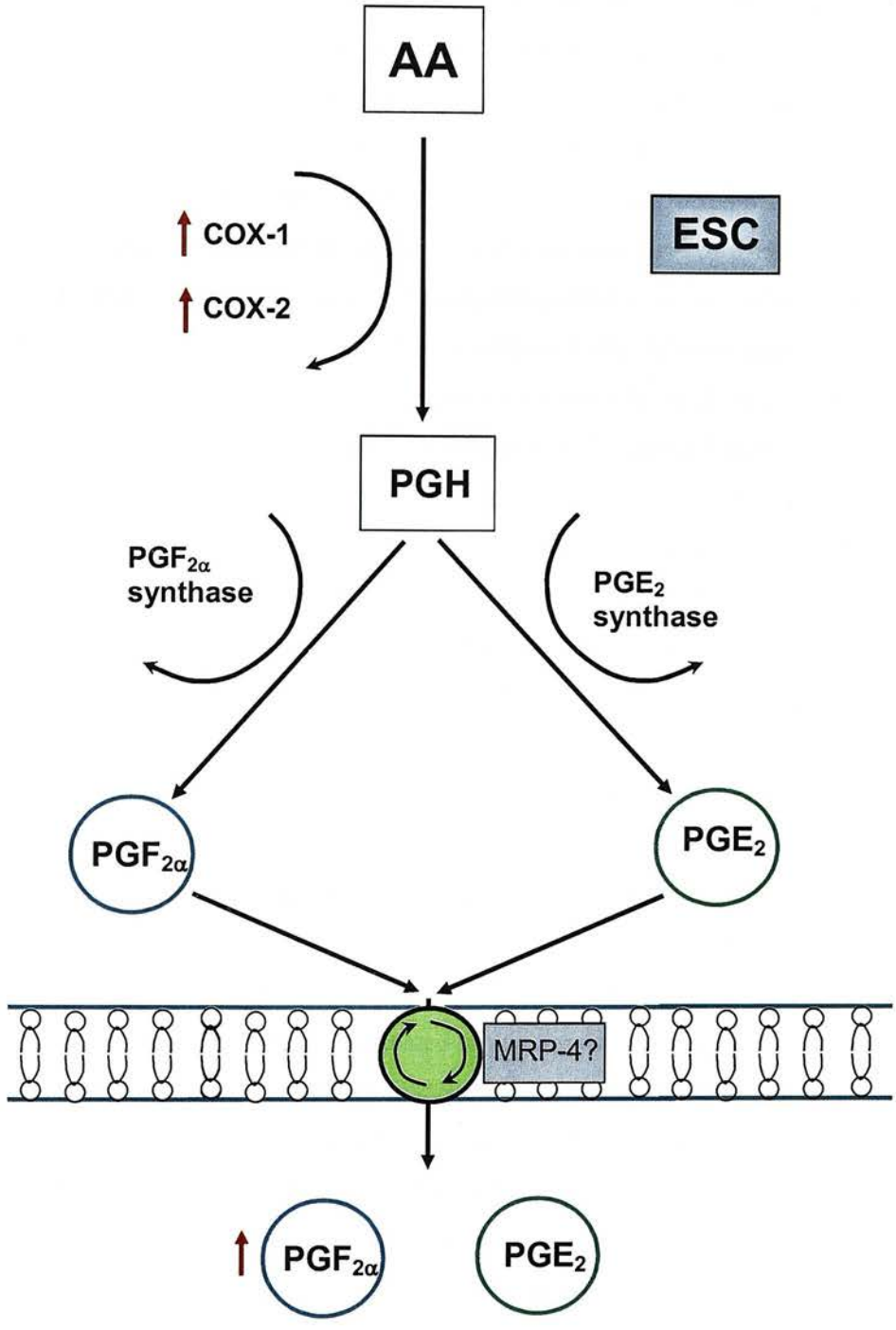
The results in figure 4.7 show no changes in the PGTP, the protein responsible for transporting PGE_2 across the cell membrane into cells, occurred in ESCs upon decidualising treatment administration. This suggests that there was no autocrine feedback to stimulate the uptake of PGE_2 upon decidualisation. The ATP-binding cassette (ABC) transporter family of proteins is involved in active transport of molecules across membranes. A sub-family of this transporter are the multi-drug resistant proteins (MRP) and MRP-4 has been implicated in the transport of PGE_2 and $PGF_{2\alpha}$ (Reid *et al.*, 2003). These transporters facilitate the movement of molecules out of cells and would be of interest to study in the context of the control of PGE_2 secretion from decidualising ESCs (figure 4.14).

The production of PGE_2 and $PGF_{2\alpha}$ has been analysed in this chapter via levels of secretion. The experiments revealed that in culture, ESCs are secreting both PGE_2 and $PGF_{2\alpha}$ although levels of PGE_2 are about 4 times greater. Also, decidualising stimuli had no appreciable effects on PGE_2 secretion levels. However, PGE_2 alone triggered a significant rise in PGE_2 secretion and MPA appeared to inhibit this response to some extent but this was not significant. Both 8-Bromo cAMP and PGE_2 did however give rise to a moderate and significant rise in the secretion levels of $PGF_{2\alpha}$ from the ESCs. This suggests that the actions of PGE_2 may be due to a rise in IC cAMP via stimulation of either or both of the EP_2 and EP_4 receptors. It is possible that the $PGF_{2\alpha}$ synthases are being stimulated causing a rise in $PGF_{2\alpha}$ production or that $PGF_{2\alpha}$ is being selectively secreted. Previously it has been demonstrated that cultured bovine luteal cells secrete

raised levels of $PGF_{2\alpha}$ following PGE_2 treatment (Del Vecchio *et al.*, 1995) confirming the ability of PGE_2 to modify the $PGF_{2\alpha}$ pathway. The functions of $PGF_{2\alpha}$ in the human endometrium are not understood. It is however known that human secretory endometrium secretes greater amounts of $PGF_{2\alpha}$ than proliferative endometrium as shown by explant culture experiments (Abel *et al.*, 1980) and that $PGF_{2\alpha}$ concentrations are greatest in late secretory endometrium (Downie, 1974; Maathuis *et al.*, 1978; Ishihara *et al.*, 1986). These findings are consistent with the proposed function for $PGF_{2\alpha}$ in the human endometrium as vasoconstrictor in uterine vessels in the pre-menstrual phase (Baird *et al.*, 1996). In menorrhagic women, endometrial concentrations of PGE_2 and $PGF_{2\alpha}$ during the luteal phase were found to be raised in almost half of the women studied compared with healthy controls (Haynes *et al.*, 1980) and this is likely to be due to a raised availability of AA in the menorrhagic endometrium (Kelly *et al.*, 1984). It is however, the ratio of $PGF_{2\alpha}$ to PGE_2 that is important in the luteal phase in the control of excessive blood loss in women since menorrhagia is associated with a shift in endometrial conversion of PGH from $PGF_{2\alpha}$ to PGE_2 (Smith *et al.*, 1981).

Figure 4.14

A summary of the alterations observed in the PG pathway by MPA, PGE_2 and a cAMP analogue (inducers of *in vitro* decidualisation) using the results of this chapter (section 4.3). These modifications are depicted by red arrows.


AA
ESC
COX-1
COX-2
PGH
PGF2α synthase
PGE2 synthase
PGF2α
PGE2
MRP-4?
PGF2α
PGE2

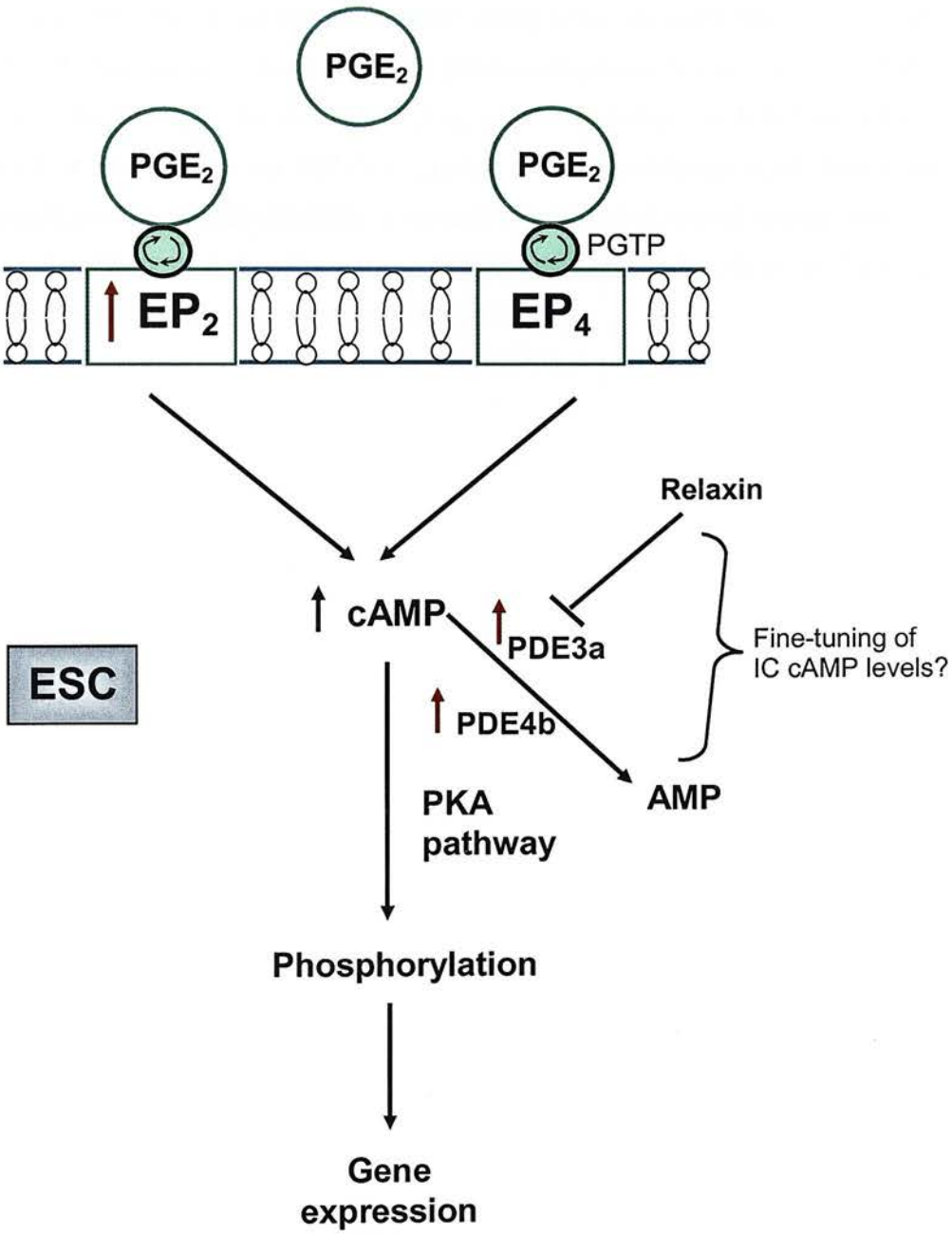
A divergent control over the EP receptors, EP_2 and EP_4, was revealed by the results detailed in this chapter. Decidualising stimuli were able to significantly raise EP_2 receptor mRNA expression levels on all days. This is in agreement with gene array data showing the EP_2 receptor is raised during the implantation window compared with the proliferative phase in human endometrium (Kao *et al.*, 2002). In contrast, the data in this chapter showed EP_4 receptor expression levels to be unaffected by the decidualising treatments with the exception of on day 4 with administration of 8-Bromo cAMP plus MPA. In order for PGE_2 to exert its decidualising effects via the cAMP pathway these receptors need to be present in the ESCs (see figure 4.15). EP_2 receptor null mice have compromised fertility, manifested as a reduced litter size (Kennedy *et al.*, 1999; Tilley *et al.*, 1999). However, decidualisation was normal and the lowered litter size found to the result of an impaired ovulatory process (Matsumoto *et al.*, 2001). It is possible that the EP_4 receptor, under these circumstances, can compensate partially for the loss of EP_2 receptor in a similar manner that COX-2 compensates for COX-1 deficiency in COX-1 -/- mice (Reese *et al.*, 1999). It is also important to consider the differences between the decidualisation process in humans and mice. In humans the process begins in the non-pregnant endometrium in anticipation of pregnancy and is essential to implantation of the embryo. However, in mice the process only occurs as a result of implantation.

A rise in IC cAMP levels and activation of the PKA pathway results in an increase in PDE activity and cAMP degradation via a "short-loop" feedback system in rat heart and fat cells (Gettys *et al.*, 1987) and in rat hepatocytes (Corbin *et al.*, 1985). The cGMP-inhibited PDE, PDE3, has been implicated in this regulatory mechanism after studies on human platelets (Grant *et al.*, 1988; Macphee *et al.*, 1988). PDE3a has been located in rat genitourinary smooth muscle and epithelial cells by *in situ* hybridisation (Reinhardt *et al.*, 1995). The atomic structure of the cAMP-specific PDE4b has been established (Xu *et al.*, 2000) and has been located in human myometrium by mRNA studies (Leroy *et al.*, 1994; Leroy *et al.*, 1999). In this chapter the relative mRNA expression levels of PDE3a and PDE4b in response to decidualising stimuli were analysed. Modest elevations in the expression of both PDE3a and PDE4b were observed in the

decidualising ESCs (Figure 4.15). Experiments on human ESCs demonstrated a rise in intracellular cAMP levels during PGE_2 plus progesterone-induced decidualisation *in vitro* and addition of a PKA inhibitor blocked this cAMP elevation (Brar *et al.*, 1997). The results in this current chapter illustrate how cAMP levels may be "fine-tuned" so that levels do not become excessively raised (see figure 4.15). Decidualisation is being stimulated and influenced by many factors and a further candidate is relaxin. In human ESC cultures, relaxin has been shown to induce expression of the decidualisation marker, prolactin (Huang *et al.*, 1987; Zhu *et al.*, 1990; Tabanelli *et al.*, 1992; Tseng *et al.*, 1999). In addition, cultured human endometrial cells treated with relaxin showed increased intracellular cAMP levels and this was enhanced by PGE_2 and $PGF_{2\alpha}$ (Fei *et al.*, 1990) and this elevation in response to relaxin is acute and permanent (Telgmann *et al.*, 1998). These effects of relaxin have been proposed to be via an inhibitory control over PDE activity (Bartsch *et al.*, 2001), although the specific PDE being regulated are as yet unconfirmed. The *in vivo* expression of relaxin has been identified in the human endometrial stroma by immunohistochemistry (Bryant-Greenwood *et al.*, 1993) (refer to figure 3.2 in chapter 3). Since relaxin acts to raise intracellular cAMP levels by inhibition of PDE activity, this is an additional stimulus on cAMP levels and therefore it will be essential that a regulatory feedback mechanism will be involved to control cAMP levels (figure 4.15). The increase in PDE3a and PDE4b levels upon raised intracellular cAMP levels, as demonstrated by the results in this chapter, could be providing some of this regulation but other PDEs not explored in this chapter could also be involved.

Figure 4.15

An illustration of the potential mechanisms by which PGE_2 may be exerting paracrine effects on ESCs and how intracellular cAMP levels may be regulated during decidualisation. The modifications in this pathway that have been explored in this chapter are highlighted by red arrows.


PGE2
PGE2
PGE2
PGTP
EP2
EP4
Relaxin
cAMP
PDE3a
Fine-tuning of
IC cAMP levels?
ESC
PDE4b
AMP
PKA
pathway
Phosphorylation
Gene
expression

The results detailed and discussed in this chapter give an insight into aspects of the PG pathway that are modified during the decidualisation process of human ESCs *in vitro*. This provides information on the intracellular changes that decidualising cells *in vitro* undergo. The fine-tuning of some of the PG pathway stages has been addressed here but gives rise to further questions and also highlights other important mediators to explore. These include how the external PGTP is regulated and this knowledge would help in the understanding of how PGE_2 secretion is controlled and how it can be upregulated. In addition, the effects of decidualisation on PGES expression and activity would be of interest as a further controlling factor in PGE_2 production.

5. IL-15 Regulation in the Human Endometrium

5.1 Introduction

Cellular interactions and cytokine production within the human endometrium are critical to the key events of menstruation, implantation and placentation. Cytokines allow communication between different cell types at the various cycle stages. The major leukocyte population in the late secretory phase and throughout the first trimester of pregnancy is the $CD56^{bright}$ $CD16^{-}$ uterine Natural Killer (uNK) cell. NK cells are absent in IL-15 (-/-) knockout mice implying a role for IL-15 in the development and maturation of these cells (Kennedy *et al.*, 2000). In the human endometrium, *in vitro* experiments on isolated human uNK cells demonstrated the ability of IL-15 to stimulate proliferation of these cells (Verma *et al.*, 2000). During the human "implantation window" IL-15 and the α chain of the IL-15 receptor are upregulated at least 3-fold compared with the late proliferative phase (Kao *et al.*, 2002) and supports the role of IL-15 in the process of implantation. In human peripheral blood at least, IL-15 has been shown to be essential for the production of type 2 cytokines, such as IL-10, by this sub-type of NK cell (Cooper *et al.*, 2001a).

Localisation of IL-15 within the human endometrium has been confirmed using immunohistochemistry (Kitaya *et al.*, 2000). During the proliferative phase of the cycle staining is highest in the glands with stromal staining in the perivascular region appearing in the secretory phase. The mechanism by which IL-15 is regulated in epithelial cells has not been explored to date. This chapter will examine, briefly, this issue using two endometrial epithelial cell lines, HES and Ishikawa. The immunoreactivity for IL-15 persists in both glands and stroma into the first trimester of decidua. Treatment of human endometrial stromal cells (ESCs) and decidual cells with progesterone stimulated the release of IL-15 *in vitro* (Kitaya *et al.*, 2000; Okada *et al.*, 2000). These results suggest hormonal control of IL-15 mRNA expression and protein release. Progesterone levels increase across the menstrual cycle, peaking at day 21 before either being maintained at a high level due to continued secretions from the corpus luteum (CL) or declining as a result of its demise in the absence of pregnancy.

Due to the timing of IL-15 appearance in the stromal compartment of endometrium and the *in vitro* influence of progesterone, the *in vivo* trigger of decidualisation, a relationship between IL-15 and the process of decidualisation has been previously proposed (Okada *et al.*, 2000a). An effective inducer of *in vitro* decidualisation is a combination of the synthetic progestin, medroxyprogesterone acetate (MPA), together with cAMP (Tang *et al.*, 1994; Brosens *et al.*, 1999). This second messenger is downstream in the PGE_2 pathway, a compound also shown to induce stromal differentiation (Frank *et al.*, 1994), and cAMP levels can be upregulated by PGE_2 via the EP_2 and EP_4 receptors (Narumiya *et al.*, 1999).

Studies in mice have identified uNK cells as a source of the T helper-1 (Th-1) cytokine, IFN-γ (Ashkar *et al.*, 2000). Further experiments involving knockout mice lacking IFN-γ (IFN-$\gamma^{-/-}$) demonstrated that the normal pregnancy-associated modifications of the blood vessels failed to occur (Ashkar *et al.*, 1999). In addition to a role in decidual artery remodelling, it was established that this absence of IFN-γ leads to a necrotic decidua in late gestation implying a role in maintenance of decidual integrity.

This chapter details the *in vitro* control of IL-15 mRNA expression and secretion by IFN-γ and PGE_2 in ESCs and examines the expression of IL-15 and IFN-γ across the menstrual cycle. In addition, a model of progesterone maintenance *in vivo* has been used to study the influence of progesterone on IL-15 immunostaining in the human endometrium.

5.2 Materials and Methods

5.2.1 Human Uterine Tissue Collection

Endometrial (n = 68) and first trimester decidual biopsies (n = 8) were collected and processed as described earlier (section 2.1). The samples used in this chapter are detailed in Table 5.2.

Table 5.2

Cycle stage	Number of biopsies
Proliferative	17
Early secretory	16
Mid secretory	9
Late secretory	7
Menstrual	9
First trimester decidua	8
Study group A: mid secretory endometrium *	5
Study group F: pregnancy simulation **	5

* ** Descriptions on the following page.

For the biopsies used in the Q RT-PCR studies to measure IL-15 and IFN-γ mRNA expression across the menstrual cycle and in first trimester decidua the total RNA was extracted and complimentary DNA prepared from 44 of the endometrial and the 8 decidual samples, utilising the methods detailed in the General Methods and Materials (sections 2.4 and 2.5). The cell separation procedure was implemented to process 14 of the endometrial biopsies into cultures of endometrial stromal cells (section 2.2).

The remaining 10 endometrial biopsies were collected as part of a study group of women:

Group (i) Normal mid secretory phase endometrium (day LH + 8-10), correlated with peak serum progesterone concentrations (mean ± SEM (nmol/l): 50 ± 11. Range: 28.7 – 91.6). This is the Control Group. (n = 5).

Group (ii) Simulation of early pregnancy (n = 5). Daily Injections of hCG were given from day LH + 8 for 14 days, thus maintaining the lifespan of the corpus luteum (CL) and therefore maintaining progesterone production. Over the 14 days incremental doses of hCG were given ranging from 125 to 20,000 IU (Illingworth *et al.*, 1990).

5.2.2 *in vitro* Primary Cell Culture Studies (ESCs)

Proceeding ESC culture preparation as described in section 2.2, cells were seeded in 12-well plates at a concentration of $1.4x10^5$ cells/ml. Three experiments were designed and performed. Treatments administered are detailed in tables 5.2, 5.3 and 5.4. In table 5.2 the time-course experiment of IL-15 mRNA expression is described. This experiment was designed to determine the ability of IFN-γ and PGE_2 to increase IL-15 mRNA levels at different time points. IFN-γ was selected as a treatment due to its ability to stimulate IL-15 expression and secretion in cortical tubular epithelial cells (Weiler 1998) and PGE_2 for its ability to stimulate decidualisation in ESCs (Frank *et al.*, 1994). There is evidence for a discordance between IL-15 mRNA expression and secretion and it is considered that post-transcriptional controls are responsible (Tagaya *et al.*, 1996). There are two splice variants of IL-15, the LSP and the SSP (refer to section 1.4.3). It is therefore important to note that the primer and probes used in the quantification of IL-15 mRNA levels by Q RT-PCR in this chapter will detect both forms of IL-15 mRNA. However, only the LSP is secreted and this form is detected in the ELISA. In addition, the same IL-15 antibody was used in the immunohistochemistry as in the ELISA and therefore the staining pattern determined within this chapter for IL-15 in the human endometrium is that of the LSP.

Table 5.2

Experiment 1: Time-course of IL-15 mRNA expression (n = 4)		
Treatment	**Concentration**	**Incubation time**
Control	N/A	0, 4, 24, 48 and 72 hours
IFN-γ	25ng/ml	4, 24, 48 and 72 hours
PGE_2	10^{-6} M	
PGE_2 + IFN-γ	10^{-6} M + 25ng/ml	

The experiment detailed in table 5.3 was designed to study the ability of decidualising treatments (see chapter 3) to stimulate IL-15 mRNA expression and secretion. In addition, IFN-γ was added to study the role of this inflammatory mediator on IL-15 production.

Table 5.3

Experiment 2: IL-15 mRNA expression and secretion (n = 3)		
Treatment	**Concentration**	**Incubation time**
Control	N/A	3, 6 and 9 days
MPA	10^{-6} M	
MPA + 8-Bromo cAMP	10^{-6} M + 0.1mg/ml	
MPA + 8-Bromo cAMP + IFN-γ	10^{-6} M + 0.1mg/ml + 25ng/ml	

Experiment 3 (see table 5.4) was designed to study in more depth the secretion of IL-15 over time.

Table 5.4

Experiment 3: IL-15 secretion (n = 7)		
Treatment	**Concentration**	**Incubation time**
Control	N/A	3 and 6 days
IFN-γ	0.1mg/ml	
MPA + IFN-γ	10^{-6} M + 25ng/ml	
8-Bromo cAMP + IFN-γ	0.1mg/ml + 25ng/ml	
MPA + 8-Bromo cAMP + IFN-γ	10^{-6} M + 0.1mg/ml + 25ng/ml	
PGE_2 + IFN-γ	10^{-6} M + 25ng/ml	
MPA + PGE_2 + IFN-γ	10^{-6} M + 10^{-6} M + 25ng/ml	

5.2.3 *in vitro* Cell Line Culture Studies (Epithelial Cell Lines)

Endometrial epithelial cells also produce IL-15 and the following experiment was designed to explore whether the regulation of IL-15 production by these cells is similar to that in ESCs. Two epithelial cell lines were used (due to the lack of availability of primary epithelial cells). The HES cell line was established from human proliferative endometrium and was found to express vimentin and cytokeratin but did not produce prolactin, a marker of ESCs (Desai *et al.*, 1994). It is critical to prevent cross-contamination from other cell lines once they are established and so they should always handled with extreme care. Previously it has been reported that the ED_{27} trophoblast-like cell line was contaminated by the WISH cell line and both were found to be genetically identical to the cell line HeLa (Kniss *et al.*, 2001). HES cells (a gift from Dr Kniss, Ohio State University, Columbus) and Ishikawa cells (a gift from Dr Nishida, University of Tsukuba, Japan) (epithelial cell lines) were cultured in 162cm^2 culture flasks at a concentration of 1.6x10^5 cells/ml in complete medium. Cells were incubated for 24 hours after passaging and seeding of cells in 12-well plates. Treatments were

then added as detailed in Tables 5.5 and 5.6 to determine whether these cells are regulated in a similar manner to ESCs.

Table 5.5

IL-15 mRNA expression in HES cells (n = 4)		
Treatment	**Concentration**	**Incubation time**
Control	N/A	48 hours
IFN-γ	25ng/ml	
8-Bromo cAMP	250μM	
IFN-γ + 8-Bromo cAMP	25ng/ml + 250μM	

Table 5.6

IL-15 mRNA expression in Ishikawa cells (n = 4)		
Treatment	**Concentration**	**Incubation time**
Control	N/A	48 hours
IFN-γ	25ng/ml	
PGE_2	10^{-6} M	
PGE_2 + IFN-γ	10^{-6} M + 25ng/ml	

5.2.4 IL-15 ELISA

At the termination of the experiments, media was collected and either stored at -20°C or assayed immediately for IL-15 in duplicate using a commercial Enzyme-linked immunoabsorbant assay. Plates were initially coated with human IL-15 capture antibody at 2μg/ml diluted in phosphate buffered saline. 100μl was added to each well. Plates were left overnight at 4°C before being washed twice in water. To each well, 100μl of Blocking and protecting medium was added and left at room temperature for 60 minutes. The blocking and protecting medium was aspirated and plates were allowed to

air dry, uncovered at room temperature before either being stored in the freezer at -20°C or used to run an ELISA.

To set-up an ELISA, plates were initially washed in water and wash buffer (dH_20 0.05% Tween; 10mM Tris; 9g/litre NaCl). Any moisture remaining in the wells was removed by suction. Each well in the plate had a final volume of 100µl. Non-specific binding was measured by providing two wells on each plate with the buffer and Tween (Per litre dH_2O: 12.11g 100mM Tris; 2g 2mg/ml BSA; 9g NaCl; 0.744g 2mM EDTA; 300µl Phenol Red Solution; 1ml preservatives; 1.5ml 20% Tween 20 Solution; pH 7.2) solution alone. A standard curve was added to each plate with a top standard of 500pg/ml. A 1:2 serial dilution was performed down to 3.9pg/ml to create eight standards in total. Between four and six replicates of a quality control with a concentration of 100pg/ml were added to enable intra-assay variability to be monitored. All test samples were run undiluted, in duplicate wells. Plates were then left overnight at 4°C.

Plates were washed three times in wash buffer and suction used to remove excess moisture. An IL-15 detection antibody was diluted to 0.1µg/ml and 100µl pipetted into each well using a multi-channel pipette. Plates were covered and secured on a plate-shaker for 90 minutes at room temperature. They were washed as before in a solution of diluted wash buffer. The streptavidin peroxidase was added at 0.125U/ml, each well receiving 100µl. Plates were sealed and placed on a plate-shaker for 20 minutes at room temperature followed by the standard washing procedure. The substrate was then mixed and 200µl transferred to each well. Each plate was allowed 20 minutes to develop and then read on a Multiscan Ex plate reader (Labsystems) using a filter with a 450nM absorbance value. The computer program Assay Zap was used to analyse the results and construct a standard curve against which the samples could be judged.

5.2.5 RNA Extraction and Q RT-PCR

The RNA was extracted and cDNA prepared from the epithelial cell line experiment and ESC experiments 1 and day 9 samples of experiment 2. IL-15 mRNA levels were measured by Q RT-PCR as described in section 2.5.

5.2.6 IL-15 Immunohistochemistry

Immunostaining for IL-15 was conducted on the study group tissues. A late secretory endometrial section was used as a positive control to ensure the procedure had worked. In addition, three extra slides from the study group tissues were included as negative controls. Slides were prepared (see section 2.7.1) and then blocked with endogenous peroxidase (3% H_2O_2) for 10 minutes. Slides were washed twice in PBS for 5 minutes each wash. An avidin-biotin step was performed using a kit (Vector, Peterborough, UK) – 15 minutes in avidin, 2 PBS washes, 15 minutes in biotin, 2 PBS washes. The first non-immune block was performed in normal horse serum (NHS) (150µl NHS diluted in 10ml PBS) for a 20 minute duration. Slides were incubated with the primary antibody for 60 minutes at 37°C. The anti-human mouse monoclonal IL-15 antibody (R&D Systems, Abingdon, UK) was diluted 1:100 and mouse IgG type1 was used as a matched negative control and diluted 1:200. After the incubation the slides were washed in PBS Tween for 5 minutes twice. Biotinylated horse anti-mouse was the secondary antibody diluted 1:200 in NHS. Slides were incubated for 30 minutes at room temperature. Slides had two 5 minute washes in PBS Tween. The tertiary complex was made up in PBS 30 minutes prior to use from an ABC kit (Vector). Slides were incubated for 30 minutes at room temperature. Slides had two 5 minute washes in PBS Tween before being stained with DAB (Dako, Cambridgeshire, UK). Slides were washed twice in dH_2O once the brown staining had appeared in the positive controls. Slides were haematoxylin counterstained for 20 seconds before being dehydrated in graded alcohols (20 seconds in each). A 5 minute step in Histoclear was followed by 5 minutes in xylene and slides were then mounted.

All samples were stained in the same immunohistochemical run to avoid artefactual variation in staining intensity. Analysis of staining within the groups was performed blindly by two individuals. Refer to section 2.7.2 for full details of this scoring system.

5.2.7 Statistical Analysis

Q RT-PCR, ELISA and immunohistochemical data were analysed by the methods described previously in section 2.8.

5.3 Results

5.3.1 Analyses Across the Menstrual Cycle

5.3.1a IL-15 mRNA Expression

IL-15 mRNA expression was detected in whole endometrial biopsies from all stages of the menstrual cycle and in whole first trimester decidual biopsies (Figure 5.1). Lowest levels were detected in the proliferative phase of the cycle. A steady rise in levels into the early and mid secretory phases was apparent with expression levels rising 3- and 6-fold respectively compared with the proliferative phase. There is a dramatic rise in the late secretory phase of approximately a 30-fold increase versus the proliferative phase and expression of significantly more IL-15 mRNA than each of the cycle phases ($p < 0.005$). Levels of expression were higher in the menstrual and decidual tissue than in the proliferative tissue by 8- and 10-fold respectively (although not significantly).

5.3.1b IFN-γ mRNA Expression

IFN-γ mRNA expression was detected in each of the cycle stages and in first trimester decidua tissue (figure 5.2). The decidual tissue was found to have the highest level of expression and this was significantly raised when compared with the early secretory phase ($p < 0.05$). The early secretory tissue expressed the lowest levels of IFN-γ and there was a gradual rise across the later cycle stages.

Figure 5.1

Relative amounts of IL-15 mRNA in proliferative (P), early secretory (ES), mid secretory (MS) and late secretory (LS) endometrium and first trimester decidua (D). Numbers in brackets indicate sample number. **a** $p < 0.005$.

Figure 5.2

Relative amounts of IFN-gamma mRNA in proliferative (P), early secretory (ES), mid secretory (MS) and late secretory (LS) endometrium and first trimester decidua (D). Numbers in brackets indicate sample number. **a** $p < 0.05$.

Figure 5.1

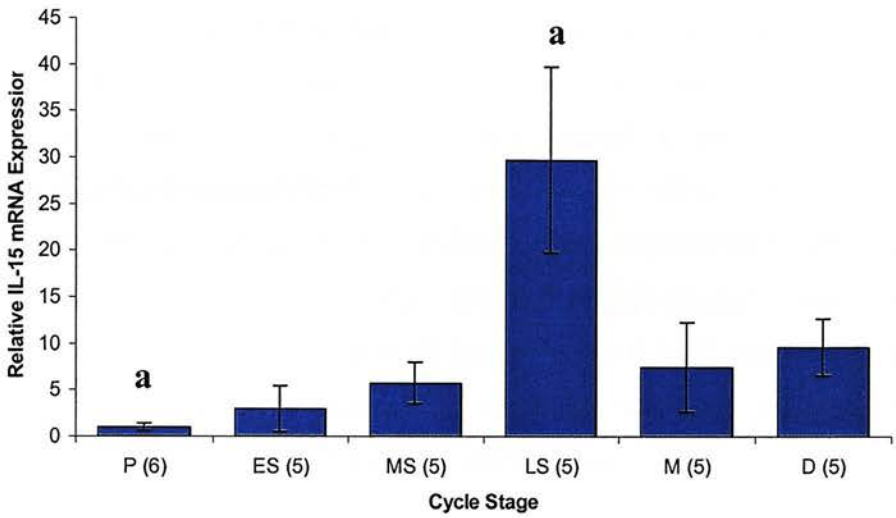
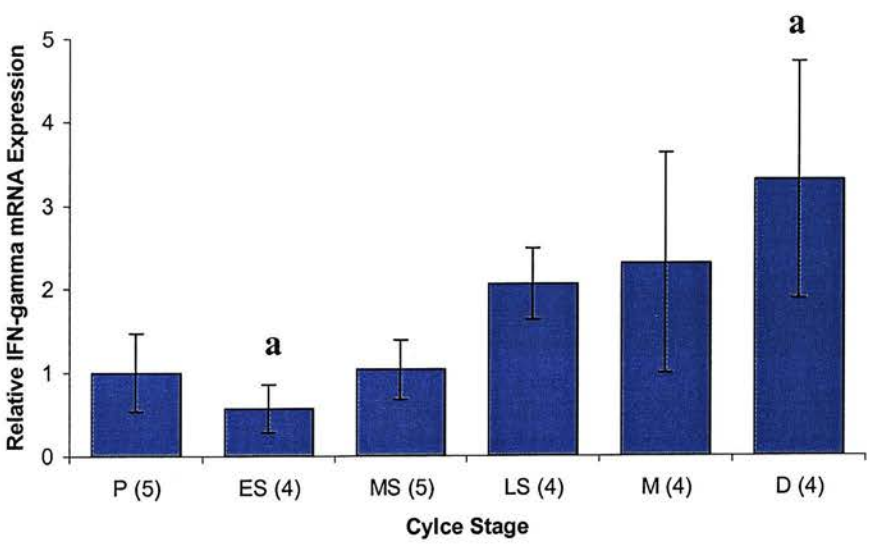


Figure 5.2



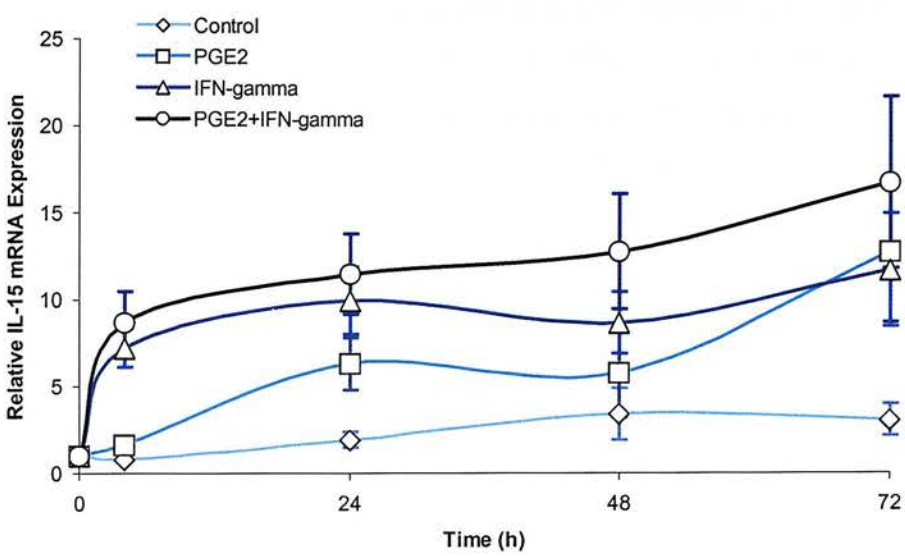
5.3.2 IL-15 mRNA Expression by Cultured ESCs

The mRNA expression of IL-15 increased throughout the time-course between 0 and 72 hours (Figure 5.3) when ESCs were treated with PGE_2 and IFN-γ alone or in combination. The control cells had consistently low levels of expression across all time points. When compared with controls the PGE_2-treated cultures by 72 hours were expressing significantly greater levels of IL-15 mRNA ($p < 0.005$). The cultures treated with IFN-γ had significantly raised IL-15 mRNA levels compared with controls at 4, 24, 48 and 72 hour time points ($p < 0.05$). The PGE_2+IFN-γ-treated cultures exhibited significantly raised IL-15 expression at all time points ($p < 0.05$) and were higher than those treated with IFN-γ alone although this difference was not significant. Refer to table 5.7 for a comparison with the epithelial cell line data.

Figure 5.3

Time course of IL-15 mRNA expression measured by Q RT-PCR in cultured ESCs (n = 4).

Figure 5.3


Control
PGE2
IFN-gamma
PGE2+IFN-gamma
Relative IL-15 mRNA Expression
25
20
15
10
5
0
0
24
48
72
Time (h)

5.3.3 IL-15 mRNA Expression and Secretion by ESCs *in vitro*

Treatment of ESCs with MPA in combination with 8-Bromo-cAMP caused a 50-fold increase in expression of IL-15 above control values after 9 days of treatment ($p < 0.05$) (Figure 5.4). MPA alone had little effect on IL-15 expression. Addition of IFN-γ, MPA and 8-Bromo-cAMP together triggered a 60-fold increase in IL-15 expression above control levels ($p < 0.03$).

IL-15 protein levels in the ESC culture media after 3 days of treatment were no different to controls when treated with either MPA alone or together with 8-Bromo cAMP (Figure 5.5). Cells treated with MPA, 8-Bromo-cAMP plus IFN-γ showed a 10-fold increase in IL-15 secretion relative to controls ($p < 0.0001$). After 6 days of treatment a similar pattern was apparent with MPA, 8-Bromo-cAMP plus IFN-γ stimulating an 11-fold increase in IL-15 protein secretion in comparison with controls ($p < 0.0003$) (Figure 5.6).

Figure 5.4

IL-15 mRNA expression measured by Q RT-PCR in cultured ESCs following 9 days of treatment (n = 3). Same letters denote significant difference. **a** $p < 0.05$ and **b** $p < 0.03$.

Figure 5.4

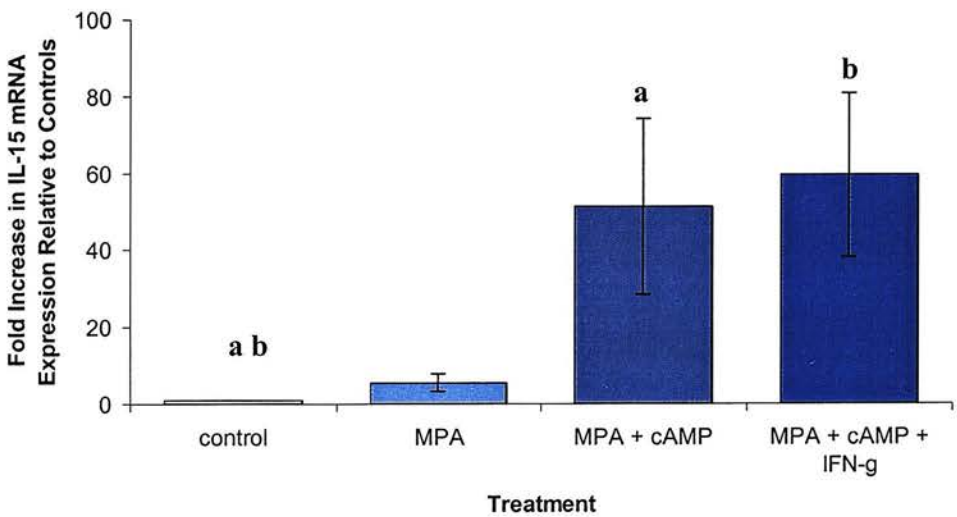

Fold Increase in IL-15 mRNA Expression Relative to Controls
100
80
60
40
20
0
a b
a
b
control
MPA
MPA + cAMP
MPA + cAMP + IFN-g
Treatment

Figure 5.5

IL-15 protein release from treated ESCs with and without IFN-γ after 3 days of treatment. (n = 3). Same letters denote significant difference. **a** $p < 0.0001$. The lower detection limit of the ELISA was 3.9pg/ml.

Figure 5.6

IL-15 protein release from treated ESCs with and without IFN-γ after 6 days of treatment. (n = 3). Same letters denote significant difference. **b** $p < 0.0003$. The lower detection limit of the ELISA was 3.9pg/ml.

Figure 5.5

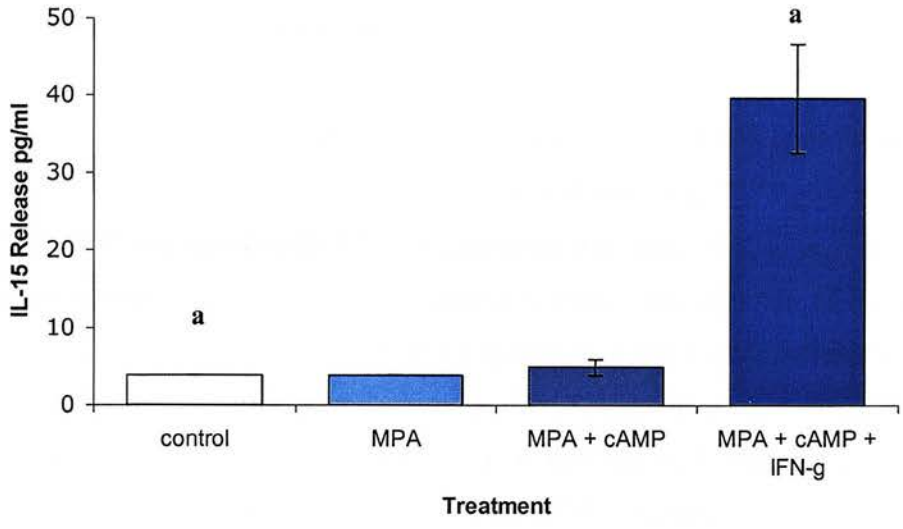
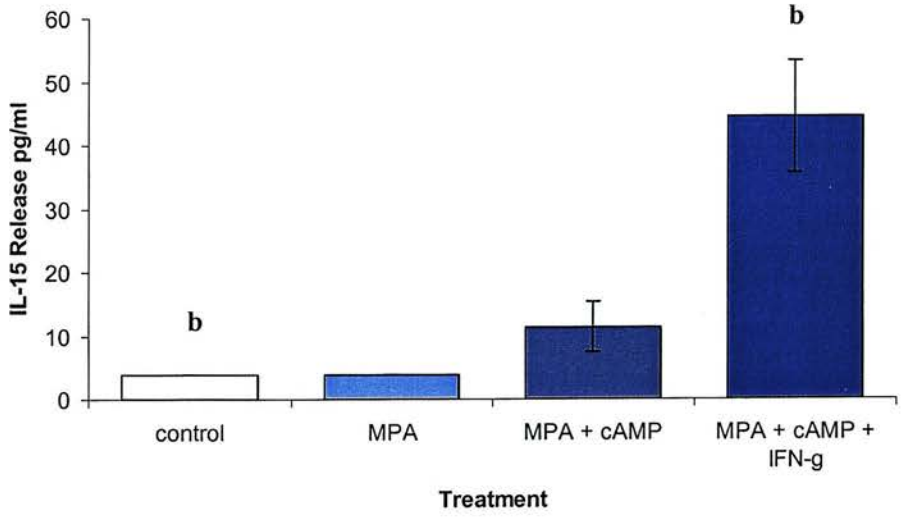


Figure 5.6



5.3.4 IL-15 Secretion by ESCs *in vitro*

As predicted from previous results (section 3.3.3) addition of IFN-γ alone stimulated a rise in IL-15 release from ESCs relative to control values ($p < 0.05$) (Figure 5.7). When 8-Bromo cAMP was added with IFN-γ a significant rise in IL-15 release was observed and this rose further by 6 days of treatment ($p < 0.01$). The addition of MPA to the treatment regime appeared to augment this response at 3 and 6 days, although this was not significant. ESCs cultured in the presence of PGE_2 and IFN-γ secreted greater levels of IL-15 relative to controls on days 3 and 6 but this only became a significant difference when MPA was supplemented ($p < 0.05$). Addition of MPA and IFN-γ stimulated a similar level of IL-15 release to treatment with IFN-γ alone.

Figure 5.7

IL-15 protein release from IFN-γ treated human ESCs after 3 and 6 days of treatment. (n = 7) * Depicts group with no IFN-γ stimulation. Same letters denote significant difference. **a, d** $p < 0.01$; **b, e** $p < 0.0002$; **c, f, g** $p < 0.05$. The lower detection limit of the ELISA was 3.9pg/ml.

Figure 5.7

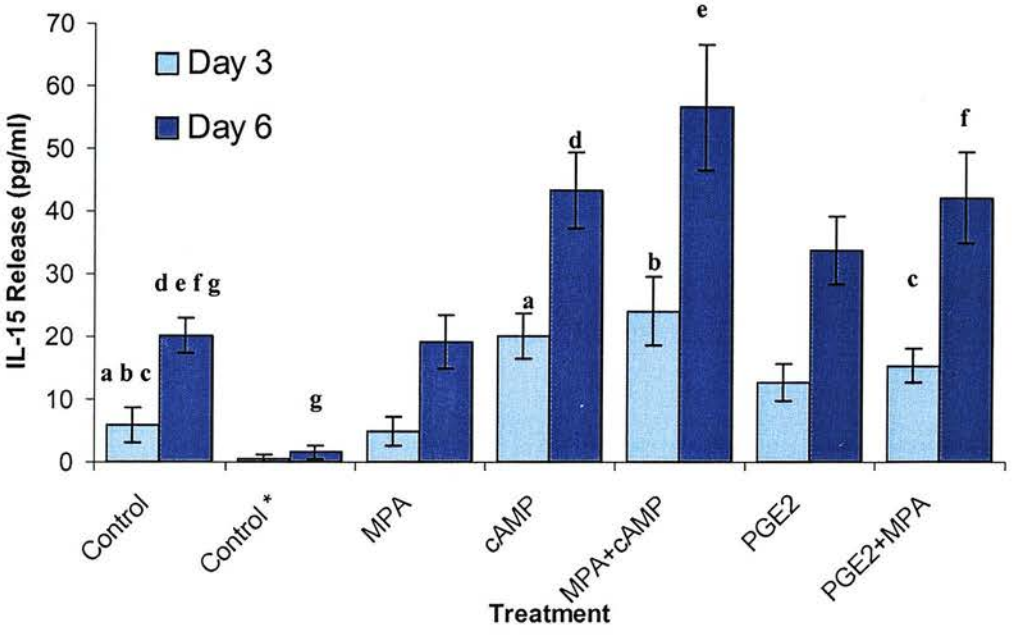

IL-15 Release (pg/ml)
70
60
50
40
30
20
10
0
Day 3
Day 6
a b c
d e f g
g
a
d
b
e
c
f
Control
Control *
MPA
cAMP
MPA+cAMP
PGE2
PGE2+MPA
Treatment

Figure 5.8

IL-15 mRNA expression measured by Q RT-PCR in cultured HES cells following 2 days of treatment (n = 4). IL-15 mRNA levels were analysed by Q RT-PCR after 48 hours of treatment. IFN-γ and 8-Bromo cAMP alone or in combination had no effect on the level of IL-15 mRNA relative to control values. (See table 5.7 for a comparison with ESC culture data).

Figure 5.8

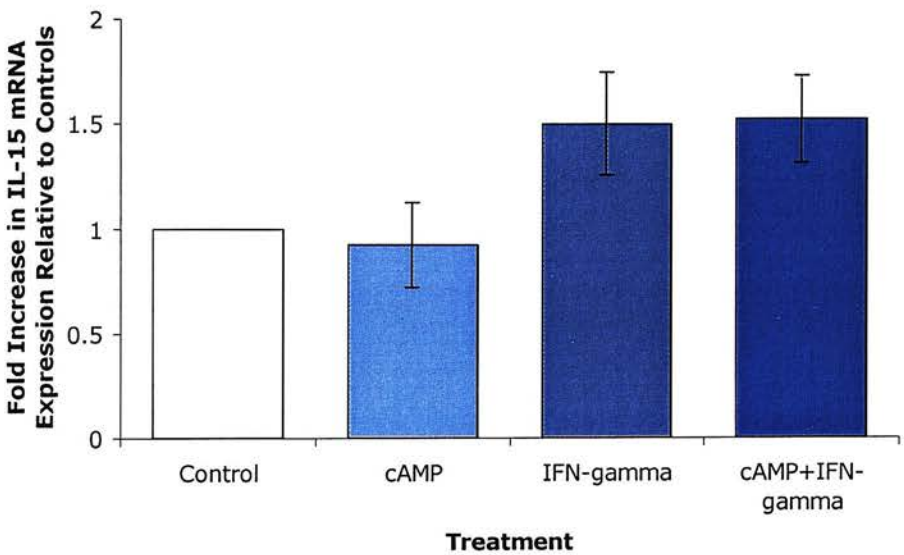
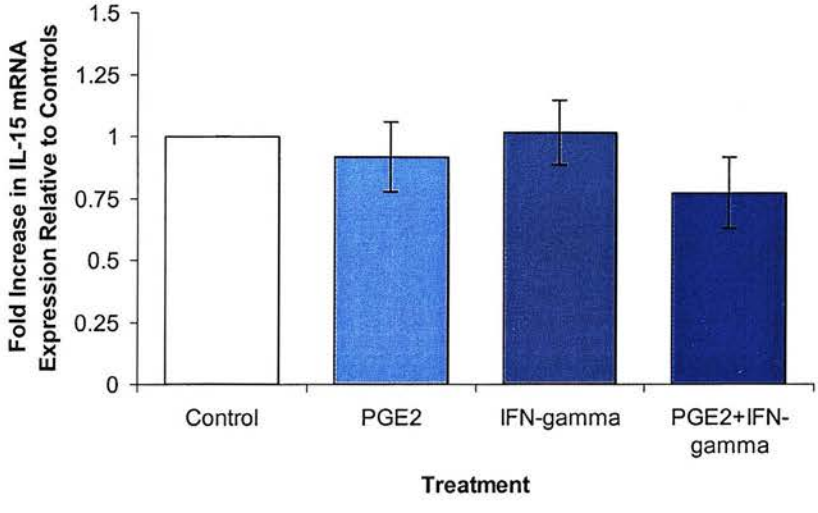

Fold Increase in IL-15 mRNA Expression Relative to Controls
2
1.5
1
0.5
0
Control
cAMP
IFN-gamma
cAMP+IFN-gamma
Treatment

Figure 5.9

IL-15 mRNA expression measured by Q RT-PCR in cultured Ishikawa cells following 2 days of treatment (n = 4). IL-15 mRNA levels were analysed by Q RT-PCR after 48 hours of treatment. IFN-γ and PGE_2 alone or in combination had no effect on the level of IL-15 mRNA relative to control values. (See table 5.7 for a comparison with ESC culture data).

Figure 5.9


Fold Increase in IL-15 mRNA Expression Relative to Controls
1.5
1.25
1
0.75
0.5
0.25
0
Control
PGE2
IFN-gamma
PGE2+IFN-gamma
Treatment

Cell type	Treatment	Fold increase in IL-15 mRNA relative to controls	*p* value
ESC	Control	1	N/A
	PGE_2	1.7	N/S
	IFN-γ	2.6	< 0.05
	PGE_2 + IFN-γ	3.8	< 0.05
HES	Control	1	N/A
	8-Bromo cAMP	N/A	N/A
	IFN-γ	1.5	N/S
	8-Bromo cAMP + IFN-γ	N/A	N/A
Ishikawa	Control	1	N/A
	PGE_2	0.9	N/S
	IFN-γ	1.0	N/S
	PGE_2 + IFN-γ	0.8	N/S

Table 5.7

Summary of comparable primary endometrial stromal cell (ESC) and epithelial cell line (HES and Ishikawa) data taken from figure 5.3, 5.8 and 5.9. All cells were treated for 48 hours.

Figure 5.10

Immunohistochemical localization of IL-15 in the normal mid-luteal (LH day 8-10) **(a and c)** and with simulation of early pregnancy **(b, e and f)**. Negative control **(d)**. A strong intensity of staining was observed in the glandular epithelium (g) of endometrium from simulated early pregnancy **(b and e)** and this was significantly greater than in the control group **(a and c)** ($P < 0.05$). The stromal cells showed no immunoreactivity in the control group **(a and c)** but endometrium from simulated early pregnancy had strong stromal (s) immunostaining **(b, e and f)**. Scale bar in photomicrograph **d** represents 200μm. Scale bar in photomicrograph **f** represents 100μm.

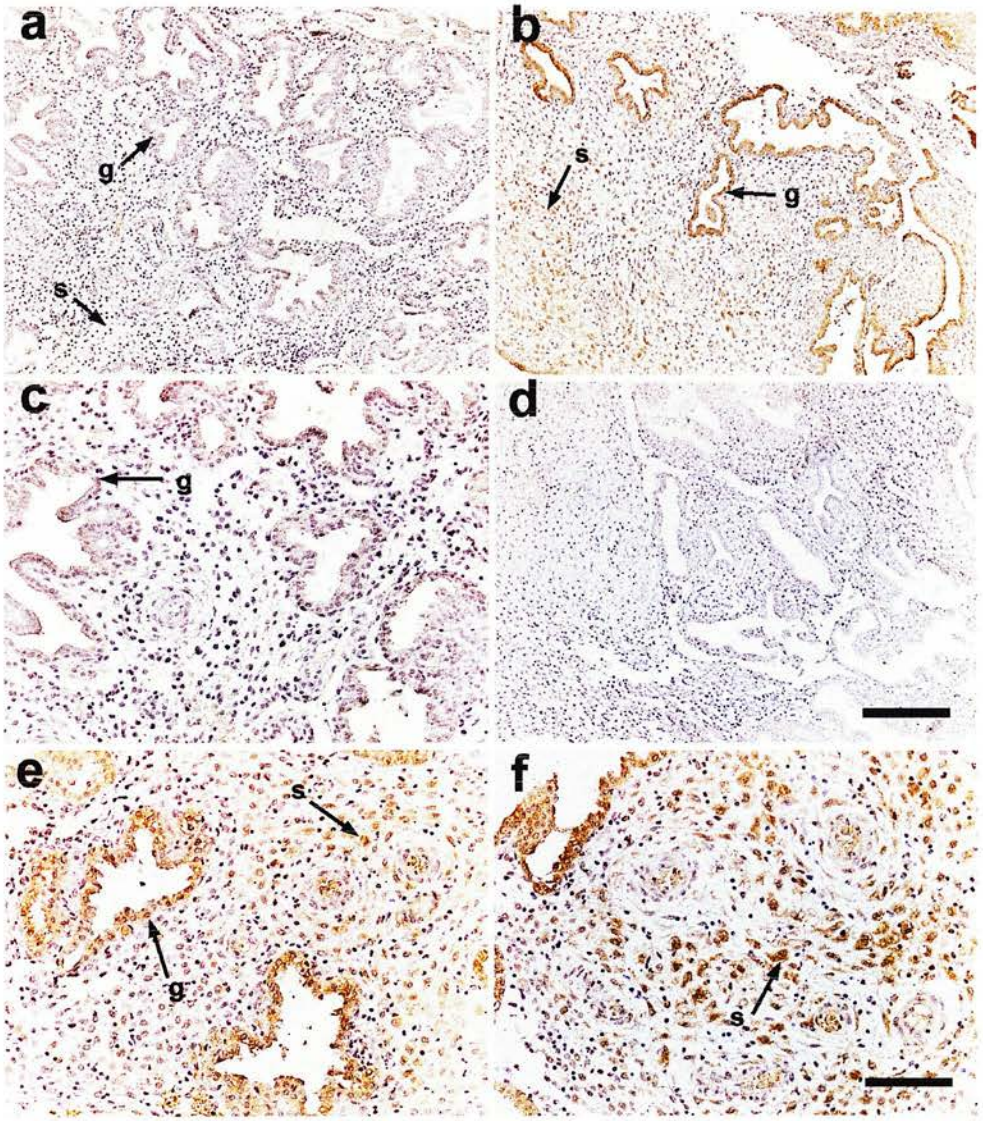
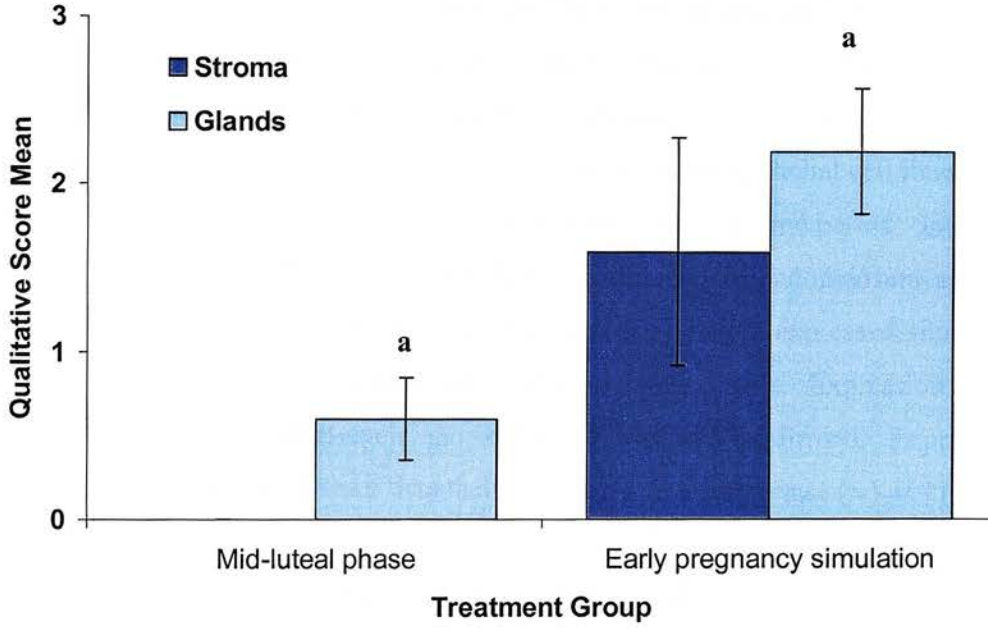

a
g
s
b
s
g
c
g
d
e
s
g
f
s

Figure 5.11

Qualitative scoring of IL-15 immuno staining in human endometrium taken from two groups of women: Normal mid-luteal (LH peak + 8-10) and simulation of pregnancy (hCG-treated for 14 days from LH + 8). Same letters denote significant difference. **a** $p < 0.05$.

Figure 5.11


3
2
1
0
Qualitative Score Mean
Stroma
Glands
a
a
Mid-luteal phase
Early pregnancy simulation
Treatment Group

5.4 Discussion

The results in this chapter demonstrated via a study simulating pregnancy that IL-15 immuno expression in the stroma and glands is increased by artificial maintenance ("rescue") of the CL. However, *in vitro* studies on isolated ESCs demonstrated that a synthetic progestin alone was not capable of increasing IL-15 mRNA expression or protein release from these cells. In contrast, both PGE_2 and 8-Bromo cAMP were able to increase IL-15 release *in vitro* in the presence of IFN-γ or in the absence of IFN-γ, a rise in IL-15 mRNA. These responses appeared to be enhanced by addition of a progestin. IFN-γ was essential for IL-15 protein secretion and when added alone, stimulated a small rise in IL-15 release from ESCs. Stimulation of ESCs with PGE_2 or IFN-γ alone or in combination, led to an increase in the expression of IL-15 mRNA *in vitro*. Despite protein detected in the glands by immunohistochemistry, an up-regulation of IL-15 mRNA expression in the HES and Ishikawa human epithelial cell lines was not achieved by cAMP, PGE_2 or IFN-γ stimulation over a 48 hour time period. In addition, expression of IL-15 mRNA in first trimester decidua and in endometrium across the menstrual cycle was demonstrated. The late secretory stages expressed significantly greater levels ($p < 0.005$) in comparison to all other cycle phases. Expression of IFN-γ mRNA across the menstrual cycle and in decidua was also confirmed. Expression in decidua was significantly greater than that in the early secretory phase ($p < 0.05$).

IL-15, a pro-inflammatory cytokine, is a potent T cell growth factor and activator and has been associated with allograft rejection (Baan *et al.*, 1998). It is a member of the four α-helix family of cytokines alongside IL-2. Although many of the effects of IL-15 and IL-2 are similar, their tissue distribution is distinct and the expression of IL-15 mRNA has been found in a large variety of cell types and tissues. The expression of IL-15 in a broad spectrum of tissue and cell types, including kidney epithelial cells (Weiler *et al.*, 1998), skeletal muscle (Quinn *et al.*, 1995) and placenta (Grabstein *et al.*, 1994) implies that the functions of IL-15 extend beyond that of the immune system. The possible role of IL-15 in uterine physiology has also recently been explored and the idea

of hormonal control over this cytokine proposed (Kitaya *et al.*, 2000; Okada *et al.*, 2000a). The involvement of IL-15 in placental establishment has also been proposed (Zygmunt *et al.*, 1998). Pre-eclampsia is associated with insufficient invasion of trophoblast cells at the time of implantation and placentation. The outcome is defective transformation of uterine spiral arteries resulting in the development of pre-eclampsia.

The results detailed in this chapter suggest that, at the intra-cellular signalling level (at least *in vitro*), the second messenger cAMP is a key regulator of IL-15 release from ESCs and that this response is likely to be linked to the action of IFN-γ. The role of IFN-γ in IL-15 release by cells has also been described previously in the kidney (Weiler *et al.*, 1998; Basok *et al.*, 2001). The mRNA expression for IL-15 is greatest in the late secretory endometrium and although the level is lower in first trimester decidua it is still 10-fold greater than in the proliferative phase. The simulation of early pregnancy study, described herein, has demonstrated a rise of IL-15 protein in the stroma. It is the LSP form of IL-15 that has been detected in the immunohistochemistry. The LSP and SSP forms of IL-15 were detected by Q RT-PCR across the menstrual cycle. It may therefore be possible that IL-15 staining would be greater in intensity and distributuion if it were possible to assess both forms with immunohistochemistry. It may be that these elevated levels of IL-15 mRNA in the late secretory endometrium represent the transformation of the stroma from a pseudo-decidualised state into fully decidualised cells, only relevant at this specific cycle stage. The presence of IFN-γ in human endometrium in lymphoid aggregates and in cells dispersed throughout the stroma has been confirmed previously by immunocytochemistry (Stewart *et al.*, 1992a). Here we have confirmed for the first time, quantitative expression of IFN-γ throughout the menstrual cycle and in first trimester decidua and demonstrated a rise in expression in first trimester decidua by Q RT-PCR. Macrophages can produce IFN-γ (Gessani and Belardelli, 1998) and uNK cells are another important source of IFN-γ and are situated both perivascularly and in close proximity to the glands (King, 2000). A study of decidual NK cells *in vitro* has demonstrated the spontaneous secretion of IFN-γ by these

cells (Ekerfelt *et al.*, 2002). There is a further infiltration of uNK cells throughout the endometrial stroma in the late secretory phase (Bulmer *et al.*, 1991). This pattern of rising uNK cell numbers post-ovulation, parallels the IL-15 expression demonstrated across the cycle in this chapter. It has been proposed that IL-15 is the stimulator of uNK cell proliferation (Verma *et al.*, 2000). These cells are believed to undergo apoptosis prior to menstruation. However, they appear to be "rescued" if pregnancy is established and full decidual transformation completed thus implying a link between uterine NK cells and decidualisation (King, 2000). Communication between the stromal cells and uNK cells appears to be important to the individual functions of these cell types (Figure 5.12).

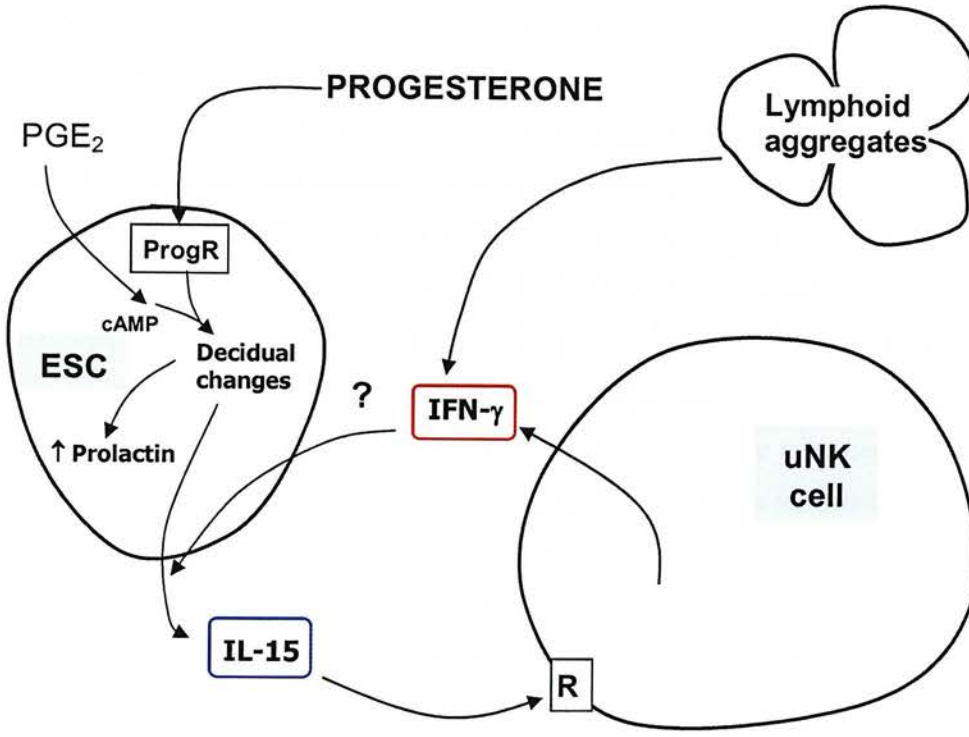


Figure 5.12

Communication between the ESCs and uNK cells could be important to the discrete functions of these cells *in vivo*. IL-15 and IFN-γ are two cytokines that may provide some of this paracrine cross-talk. **R** represents the IL-15 receptor which is composed of three subunits: the common γ chain, an α chain specific to IL-15 and the β chain of the IL-2 receptor.

In the present chapter it has been demonstrated that PGE_2 is also able to stimulate IL-15 expression and release by ESCs. The actions of PGE_2 have been established, in part, to be via the second messenger cAMP through activation of EP2 and EP4 receptors and this supports the present observations (Coleman *et al.*, 1994). The responses of both PGE_2 and 8-Bromo cAMP appear to be enhanced by addition of a progestin. This is in agreement with other studies that have suggested progesterone has an involvement in IL-15 regulation in human endometrium (Kitaya *et al.*, 2000; Okada *et al.*, 2000a) and also in concordance with IL-15 immunohistochemistry in this chapter. These data also illustrated a rise in IL-15 in the stroma and glands *in vivo*. Treatment of human epithelial cell lines with cAMP, PGE_2 or IFN-γ alone or in combination failed to stimulate an increase in IL-15 mRNA expression. Treatment of ESCs with the same regime significantly raised IL-15 mRNA expression over the same time period. These data suggest that IL-15 regulation in stromal and epithelial cells *in vitro* is taking place via divergent pathways. This is apparent according to IL-15 immunoreactivity across the menstrual cycle (Kitaya *et al.*, 2000) where in the proliferative phase the glands stain strongly for IL-15 although stromal staining is weak. The function of IL-15 in the endometrial epithelial cells has yet to be explored.

Studies using endometrial explant cultures have compounded evidence for hormonal control of PGE_2. Secretory endometrium released greater levels of prostaglandins than proliferative endometrium, corresponding with the rise in progesterone levels, and from these results an involvement of PGE_2 in menstruation has been proposed (Abel *et al.*, 1980; Baird *et al.*, 1996). Additionally, synergistic actions between MPA and oestradiol plus PGE_2 on the process of *in vitro* decidualisation have previously been reported (Frank *et al.*, 1994). An up-regulation of IL-15 mRNA expression during *in vitro* decidualisation (Okada *et al.*, 2000a) supports the link between IL-15 and decidualisation (refer to chapter 3). Previous studies have demonstrated a central role for cAMP in increasing the expression of prolactin, a marker of decidualisation (Tang *et al.*, 1993a; Brosens *et al.*, 1996). In purified blood NK cells the ability of PGE_2 to suppress the actions of IL-15 on NK cells via inhibition of the IL-15 receptor γ-chain has

been demonstrated (Joshi *et al.*, 2001). If a similar effect can be demonstrated in uNK cells this could offer a possible paracrine regulatory system to restrict uNK cell proliferation. This would be essential since women suffering from recurrent miscarriage were found to have significantly raised levels of uNK cells compared with fertile controls (Quenby *et al.*, 1999). However, these findings were discovered in the non-pregnant endometrium in the pre-implantation phase. It is not a direct reflection to what is happening in the pregnant uterus in the early stages of gestation with regard to uNK cell number and function. In contrast uNK deficient mice have small placental size (Ashkar *et al.*, 1999) that may lead to problems such as pre-eclampsia or intra-uterine growth retardation of the fetus.

In conclusion, this study provides evidence of a role for IFN-γ in IL-15 regulation in concert with the actions of PGE_2 and supports the view that IL-15 is closely linked to decidualisation and uterine NK cell function. Progesterone secretion from the CL *in vivo* increases IL-15 expression in both glandular and stromal compartments of the human endometrium although regulation of IL-15 mRNA expression *in vitro* is disparate in ESCs and human epithelial cell lines. A greater knowledge about stromal and uNK cell interactions in human endometrium and first trimester decidua will benefit our understanding of key reproductive processes such as decidualisation, menstruation, placental formation and the establishment and maintenance of early pregnancy.

6. Characterisation of Uterine Natural Killer Cells

6.1 Introduction

In the first trimester of pregnancy the major immune cell is the uterine natural killer (uNK) cell comprising approximately 70% of the bone marrow-derived cells (Loke *et al.*, 1995) and they have a specific phenotype, $CD56^{bright}$ $CD16^{-}$ (Starkey *et al.*, 1988). In a comparison of IL-10 decidual staining in missed abortion versus elective terminations, immunostaining intensity was reduced in the majority of the missed abortion cases (Plevyak *et al.*, 2002). The IFN-γ staining pattern did not differ between the two groups of women. These data suggest that the cytokine profile of uNK cells may be important in maintenance of the first trimester of pregnancy.

uNK cells respond to IL-2 (Ferry *et al.*, 1990; Nishikawa *et al.*, 1991; King *et al.*, 1992) although this cytokine has not been detected in the human endometrium or decidua (Saito *et al.*, 1993; Jokhi *et al.*, 1994b; King *et al.*, 1995). IL-15 has many functional similarities with IL-2 although its distribution is distinct with IL-15 being widely expressed (Grabstein *et al.*, 1994; Quinn *et al.*, 1995; Weiler *et al.*, 1998). The IL-15 receptor is composed of three subunits: IL-15 receptor-α (the molecule binding site), IL-2 receptor-β and the common γ chain (the signal transduction site). uNK cells have been shown to proliferate *in vitro* upon treatment with IL-15 (Verma *et al.*, 2000) and IL-15 null mice have a marked reduction in thymic and peripheral blood (PB) NK cells (Kennedy *et al.*, 2000). Knock-out mice for Interferon regulatory factor-1 (IRF-1) are unable to induce IL-15 in the bone marrow and are deficient in NK cells (Ogasawara *et al.*, 1998) implying a role for IL-15 in the survival and expansion of NK cells *in vivo*. In addition, $CD56^{bright}$ NK cells in blood have been reported to require IL-15 for their Th-2 cytokine production (Cooper *et al.*, 2001a). Cytokines released by T cells reflect the mode of dealing with disease that has been programmed into the cell and can be classified as either T helper-1 (Th-1) or -2 (Th-2), with each group exhibiting divergent roles. The Th-1 cytokines include IFN-γ, IL-12 and IL-2, and are generally classified as being pro-inflammatory whereas Th-2 cytokines, for example, IL-10 and IL-6, are considered to be immunosuppressive. However, IL-10 can also be expressed by Th-1

cells in humans but not in mice (Romagnani, 2001). Immunocompetent cells, of haematopoietic lineage, such as macrophages, T-cells and NK cells are major sources of cytokines within the uterus.

There appears to exist an intimate association between the uNK cell and endometrial stromal cells and the role of uNK cells as a pre-requisite for decidualisation in humans has been discussed (King, 2000). In mice, the uNK cell has been implicated in the maintenance of stromal decidualisation but not it's initiation (Croy *et al.*, 2002). In chapters 3 and 4 the role of PGE_2 in decidualisation has been examined. PGE_2 can modify the effects of IL-2 on PB NK cells via inhibition of the IL-2 receptor (Parhar *et al.*, 1989). Studies on mouse decidual NK cells have implicated PGE_2 in controlling their differentiation and function (Linnemeyer *et al.*, 1993). In this chapter the possible influence of PGE_2 on NK cell function in the human uterine environment will be examined.

CD40 is a member of the TNF-α receptor family and along with its ligand, CD40L (CD154), is involved in proinflammatory signalling via the NFκB transduction pathway (Berberich *et al.*, 1994). CD40 is expressed on a range of cell types including vascular endothelial cells (Hollenbaugh *et al.*, 1995) and fibroblasts (Fries *et al.*, 1995) and more recently has been localised to the stromal compartment of the human endometrium, with a focus in locality in the perivascular region (King *et al.*, 2001). It was demonstrated that treatment of stromal fibroblasts with IFN-γ and CD40L stimulated their secretion of IL-6, IL-8 (the main neutrophil chemoattractant) and MCP-1 and this may represent a role in endometrial tissue remodelling. In addition, stimulation of the CD40 system in lung fibroblasts results in raised COX-2 expression (Zhang *et al.*, 1998b) and if a similar pathway takes place in endometrial fibroblasts this pathway provides a further mechanism for inducing PGE_2 production by ESCs. IL-2-activated NK cells can express CD40L (Carbone *et al.*, 1997). uNK cells are located surrounding the blood vessels, where the CD40 protein has been localised in the stroma (King *et al.*, 2001), and therefore it may be possible that these two cell types may communicate by cell-cell

contact via the CD40-CD40L pathway. It is necessary to identify initially whether uNK cells are able to express CD40L.

Immunodeficient mice lacking uNK cells have an abnormal implantation site including acellularity of the decidua and decidual arteries with thick walls and a decreased luminal diameter (Greenwood *et al.*, 2000). Likewise, with IL-2 receptor-γ chain null mice, no uNK cells are present and these mice exhibit poor decidual transformation although they do experience apparently successful pregnancies (Miyazaki *et al.*, 2002). A link between uNK cells and decidual transformation in humans has been proposed and their decline immediately prior to menstruation has implicated them in endometrial breakdown (King, 2000). The number of uNK cells varies in a cyclical manner with a progressive rise across the secretory phase, with a marked rise at the time of implantation in the mid secretory phase (King *et al.*, 1989b) (figure 6.1). Their cyclical variation indicates they are hormonally regulated although this is likely to be indirect since they are lacking in the nuclear progesterone receptor (Henderson *et al.*, 2003). They have also been implicated in human implantation and placentation (King *et al.*, 1990; King *et al.*, 1991).

This chapter examines the effects of *in vitro* culture of uNK cells, separated from human first trimester decidua, with IL-15 and PGE_2 treatment. IL-10 production, a cytokine believed to support pregnancy, has been studied and compared with PB NK cells. Chapter 5 demonstrated the essential role for IFN-γ in IL-15-release from ESCs *in vitro*. IL-15 is a known regulator of uNK cell function and in this chapter the effects of IL-15 and PGE_2 treatments on IFN-γ and CD40L mRNA expression in uNK cells will be explored. This chapter has also assessed the effect of these treatments on the expression of two of the PGE_2 receptors, EP_2 and EP_4.

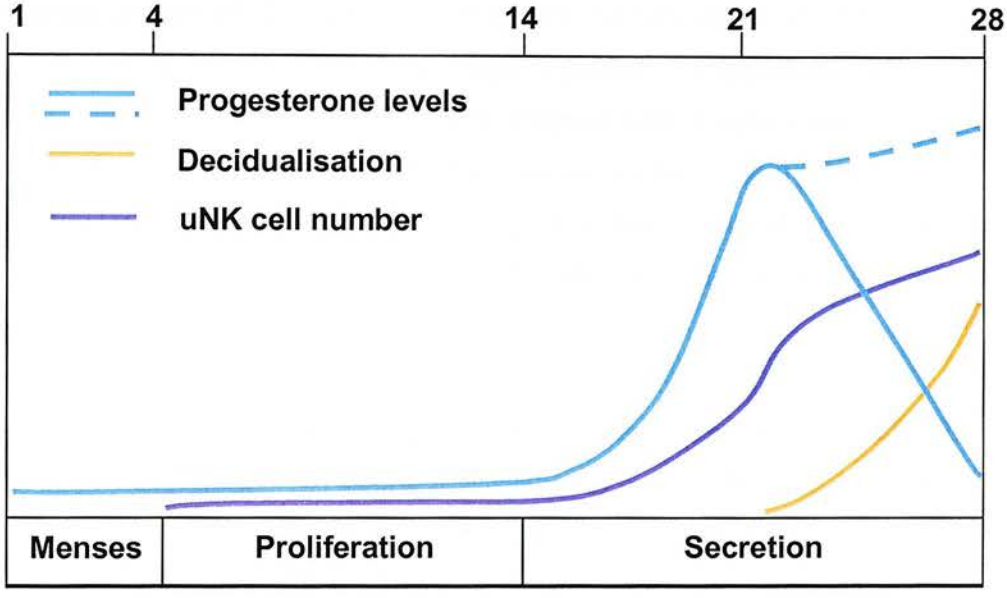


Figure 6.1

The cyclical variation in uNK cell number in relation to progesterone levels and ESC decidualisation in the human endometrium based on the "classic" 28 day cycle. The dotted blue line represents progesterone levels in the late secretory phase in the pregnant state.

6.2 Methods

6.2.1 Uterine Natural Killer Cell Separation Procedure

Samples of first trimester decidua (n = 9) were collected for isolation of uNK cells (collection details in section 2.1). The method was obtained from Professor A Moffet-King, Department of Pathology, Cambridge University, UK. Figure 6.2 is a paraffin-embedded section of this tissue and illustrates the abundance of uNK cells in first trimester decidua by the CD56 immunostaining pattern. Samples were washed in RPMI for 20 minutes upon collection and then chopped into 1-2mm pieces using scalpels (Swann-Morton, Sheffield, Yorkshire, UK) and suspended in 12ml RPMI + 10% FCS in a 50ml Falcon tube. 7.2mg of Type V collagenase (Sigma) was added to produce a final concentration of 0.6mg/ml. The tube was then incubated on a turning machine at room temperature overnight.

On the second day of the process, the falcon tube was topped up to 50ml RPMI + 10% FCS and re-suspended. The sediment was allowed to settle for 10 minutes and the top 45ml was then passed in turn through 70μm and 40μm sterile filters (Becton Dickinson, Europe, France). The cells were pelleted by spinning in a centrifuge for 5 minutes at 450xg. The supernatant was discarded and the pellet re-suspended in 50ml RPMI + 10% FCS. This suspension was divided between two 50ml Falcon tubes, each containing 15ml Histopaque 1077 (Sigma). 25ml was carefully layered on top of the Histopaque using a sterile pasture pipette. The tubes were spun at 800xg for 15 minutes at room temperature, ensuring the brake on the centrifuge was switched off. The lymphocyte band of cells was harvested and diluted in RPMI + 10%FCS at least 1:5 and spun and washed three more times for 5 minutes at 450xg in order that any Histopaque was removed. A red cell lysis buffer (RCLB) (1mM NH_4HCO_3; 114mM NH_4Cl; in distilled H_2O) was applied to the cells to remove any remaining red blood cells. 1ml of PBS was added per 1 5 10^8 total cells and 4 ml of RCLB was added per 1ml PBS. Cells were incubated on ice for 10 minutes. PBS was added to produce a 50ml volume and tubes

were spun for 10 minutes at 1200rpm in order to thoroughly wash the cells and remove the RCLB. Cells were then re-suspended in RPMI + 10 % FCS and a cell count conducted. The cells were ready for purification with CD56 positive magnetic beads.

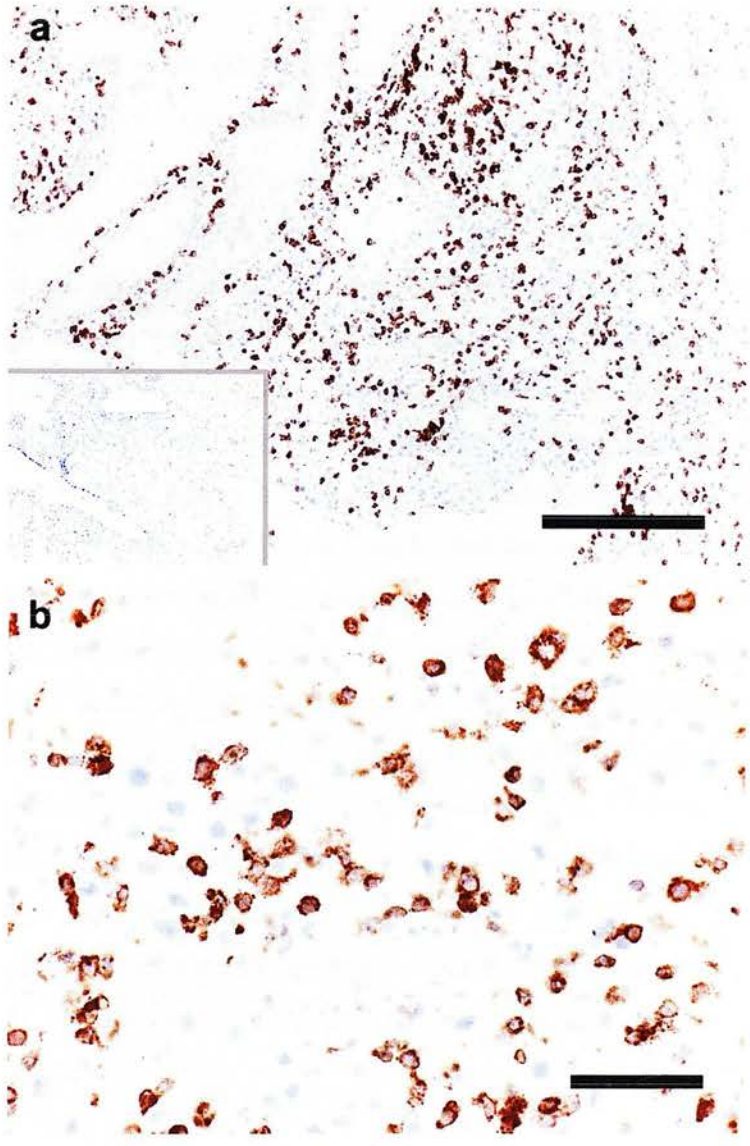


Figure 6.2

CD56 immunostaining in first trimester decidua at low power and high power magnification. Insert is the negative control. (**a**) Scale represents 200μm. (**b**) Scale represents 50μm.

6.2.2 Magnetic Bead Purification

The decidual NK cells were purified by magnetic bead isolation (Miltenyi Biotec Ltd, Surrey, UK). 100µl of CD56 MACS microbeads were added per 1 5 10^8 total cells resuspended in 300µl of CSM. The suspension was incubated for 20 minutes at 4°C and then washed in 10 ml CSM for 10 minutes at 300xg. The cells were resuspended in 500µl of CSM and applied to a LS Column attached to a VarioMACS magnet (Miltenyi Biotech). The column was removed from the magnet and washed three times and the positive cells then eluted with CSM. The purity of cells from this method of selection, as determined by FACS analysis, was on average 75% (see general methods section 2.3.1). Two aliquots of approximately 0.5 million cells was taken prior to incubation with magnetic beads, one to serve as a "blank" and the second as the negative control, and again after the positive selection procedure was completed. The "blank" tube was put through the FACS machine without any further treatment. The other two aliquots were resuspended in 200µl CSM and 10µl of anti-mouse IgG whole molecule with FITC conjugated (Sigma) added to each. The tubes were wrapped in foil and kept on ice for 30 minutes before being washed in 1ml of CSM and spun for 1 minute at 4000rpm. The cells were resuspended in 500µl of FACS buffer. Before passing the cells through the FACS machine the cell suspensions were passed though cell strainers (Becton Dickinson) to ensure a single cell suspension. For an example of the FACS recordings, see figure 6.3. The cells were then either embedded in agarose (section 6.2.4) or used in cell culture experiments (6.2.6).

Figure 6.3

A representative example of the FACS recordings for the purity of the separated uNK cells. The mouse anti-human matched negative control (**A**) and the sample incubated with the mouse anti-human CD56 antibody (**B**).

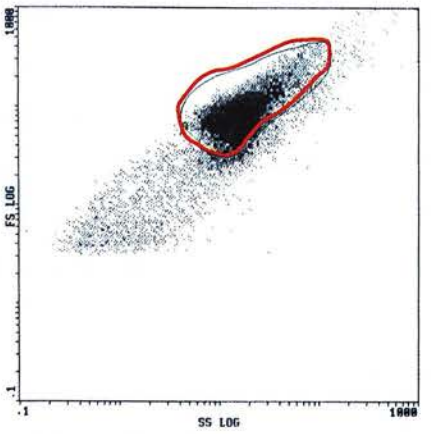
A **MRC EDINBURGH**
COULTER(R) EPICS(R) Acquisition Flow Cytometry Report

14Nov02 16:57:25
fitc 17.10.02 CD NK2
Z0013960
14.11.02 D72 PF neg fitc
48 seconds. 12084 events
Stop Count: 10000 events, histogram 3

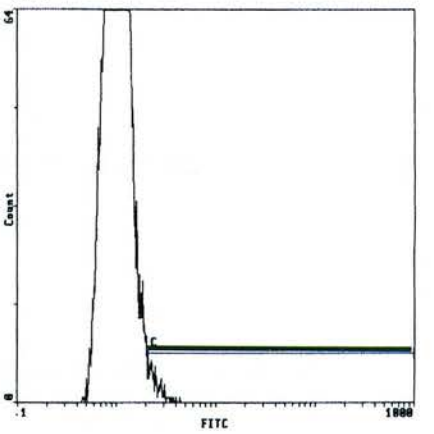
OP ID: SJD

Initial cytosett. from prot. fitc 17.10.02 CD NK2

(i)



(ii)



Stats: Normalized, Listgating: Disabled
Color equations

Hist	Region ID	%	Count	Mn X	Mn Y	PkPosX	PkPosY	PkCnt	FPCVX	FPCVY
1	A A	82.8	10000	16.4	81.0	14.7	71.5	85	52.52	43.09

Hist	Region ID	%	Count	Mn X	Md X	PkPosX	PkCnt	HPCV	Min	Max
3	C C	2.06	206	2.64	2.52	2.16	10	0.95	2.14	1024

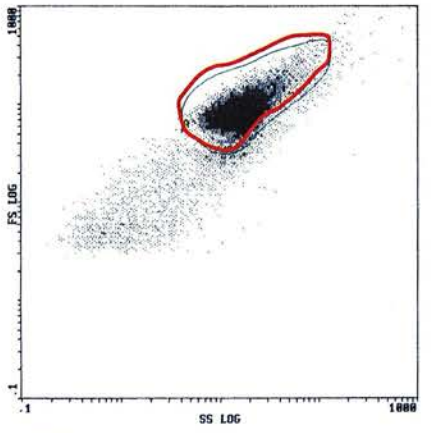
B **MRC EDINBURGH**
COULTER(R) EPICS(R) Acquisition Flow Cytometry Report

14Nov02 16:59:26
fitc 17.10.02 CD NK2
Z0013961
14.11.02 D72 PF pos fitc
48 seconds. 11809 events
Stop Count: 10000 events, histogram 3

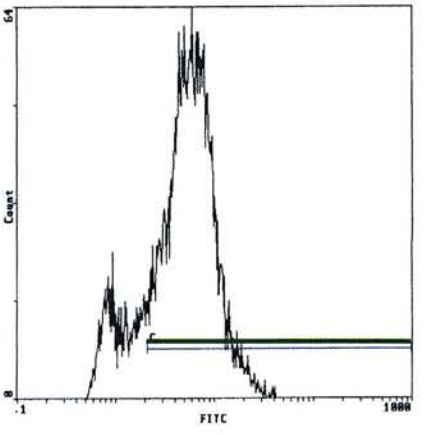
OP ID: SJD

Initial cytosett. from prot. fitc 17.10.02 CD NK2

(i)



(ii)



Stats: Normalized, Listgating: Disabled
Color equations

Hist	Region ID	%	Count	Mn X	Mn Y	PkPosX	PkPosY	PkCnt	FPCVX	FPCVY
1	A A	84.7	10000	16.7	84.0	14.7	82.5	96	50.66	39.46

Hist	Region ID	%	Count	Mn X	Md X	PkPosX	PkCnt	HPCV	Min	Max
3	C C	82.4	8243	6.15	6.06	5.12	73	28.53	2.14	1024

6.2.3 Peripheral Blood (PB) NK Cell Separation

Buffy coats were collected from healthy donors (n = 5) from the Blood Donor Centre, Edinburgh, UK. The blood was diluted 1:4 with PBS and the peripheral blood monocyte cells (PBMC) were separated out using Histopaque 1077 as detailed above in section 6.2.1. CD56 positive NK cells were purified using the method described in 6.2.2 and FACS (section 2.3 and 2.3.1) determined the purity of separation to be > 90%.

6.2.4 Agarose Embedding

The negative and positive column fractions from the uNK cell separation were retained in order to make comparisons. Aliquots of 0.5 – 1 million cells were taken and washed in 1 ml of PBS for 1 minute at 4000 rpm. The supernatant was removed and 200µl of NBF added to briefly fix the cells. Tubes were centrifuged at 1500 rpm for 10 minutes. The supernatant was discarded and cells were resuspended in 10µl PBS. Agarose gel (2% agarose MP (Sigma) in PBS) was pipetted onto glass slides in the confinement of a circle drawn with a hydrophobic pen (Sigma). Before the agarose gel had set, the 10µl cell suspension was pipetted just below the surface of the gel. The piece of gel was wrapped in speci-wrap that had been pre-soaked in 70% ethanol and submerged in a vial of 70% ethanol. Samples were then processed for wax embedding and sections were cut and either processed for H and E staining (figure 6.4) or for CD56 immunohistochemistry.

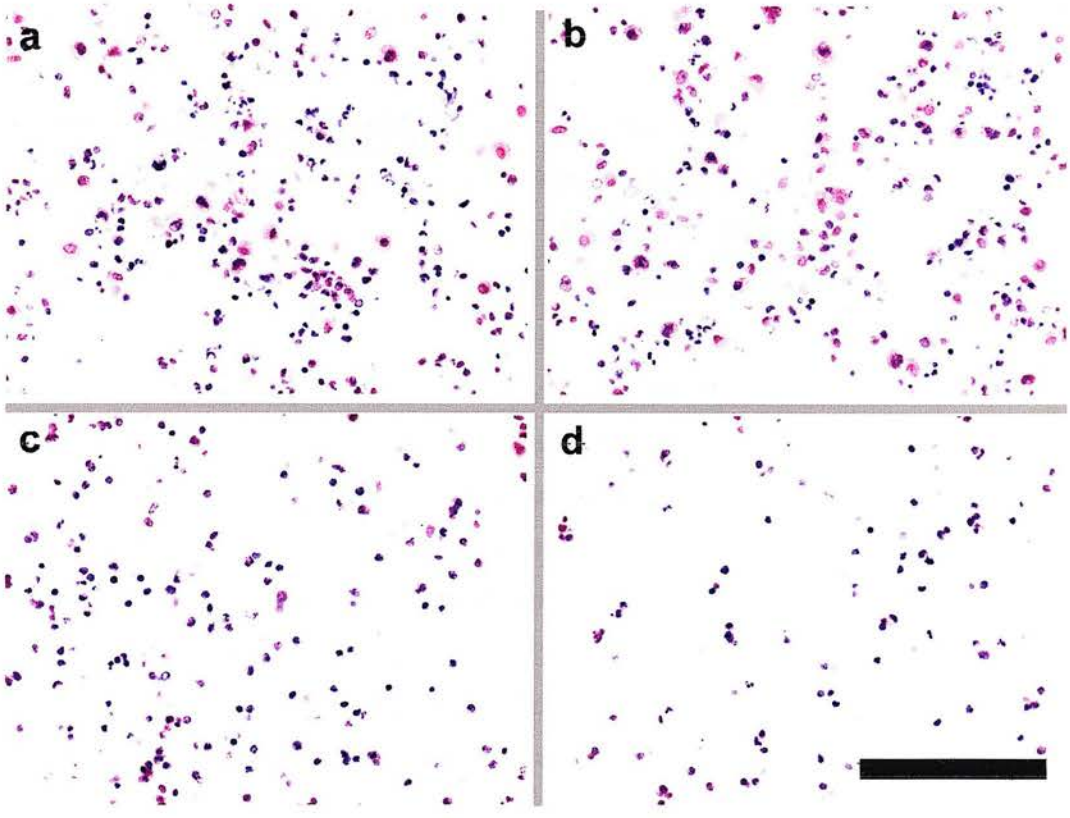


Figure 6.4

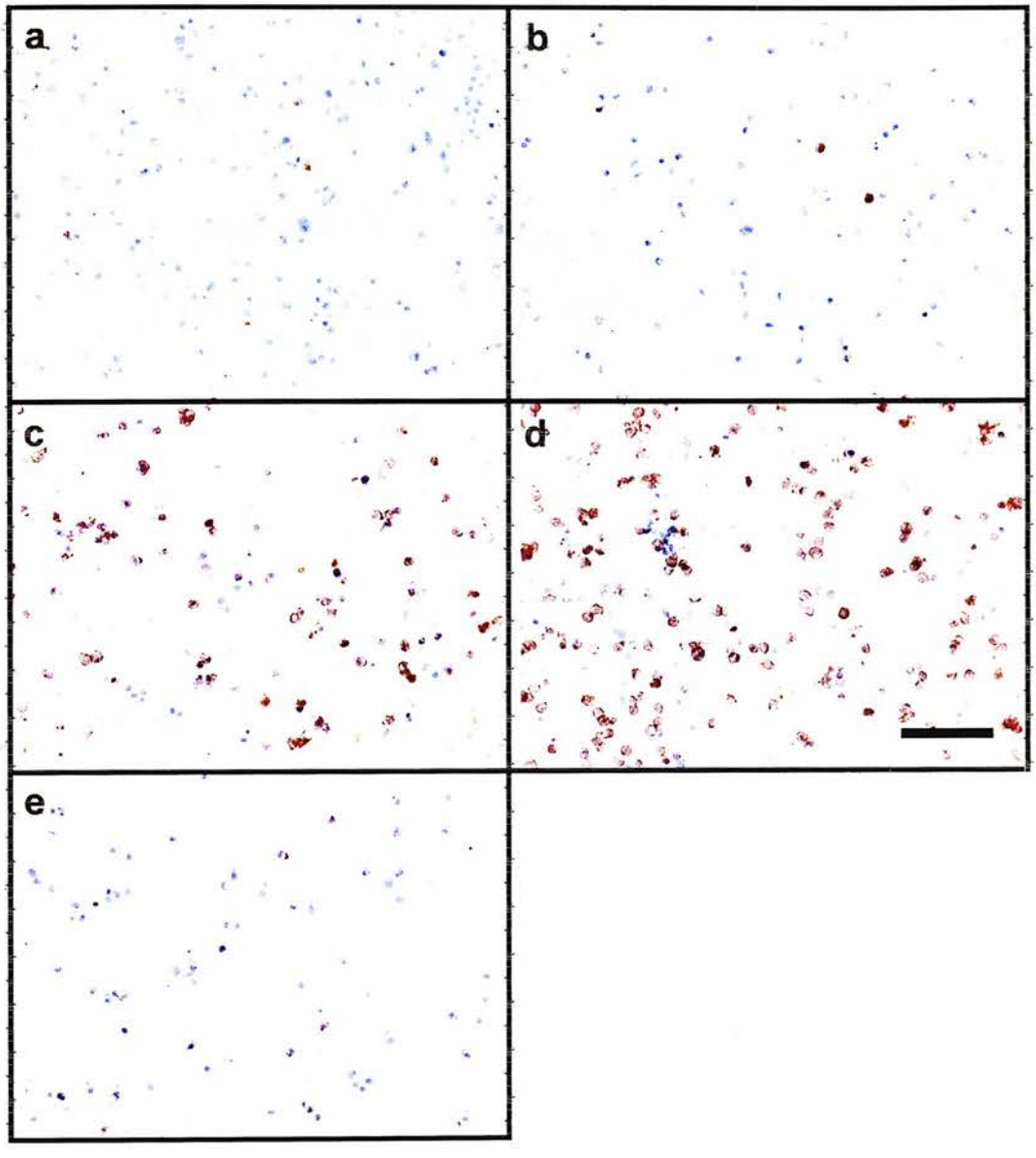
Photomicrographs of haematoxylin and eosin stained decidual cells: positive and negative column fractions. (**a** and **b**). Cells from the negative column fraction. (**c** and **d**). Cells from the positive selection column fraction. Scale bar represents 100μm.

6.2.5 CD56 Immunohistochemistry

Slides were prepared as in section 2.7.1 (except TBS was used in place of PBS) and then placed in a pressure cooker for 5 minutes in a solution of 0.01M sodium citrate pH6. This antigen retrieval step would break the cross-links formed during the fixation process and allow antibodies to access the previously masked epitopes on the protein of interest. Endogenous peroxidase activity was blocked with hydrogen peroxide (3% H_2O_2) for 30 minutes, followed by two 5 minute washes in TBS. Slides were blocked with diluted NHS (a non-immune block) (150µl NHS diluted in 10ml PBS) for 20 minutes at room temperature, followed by a 60 minute incubation at 37°C with the mouse anti-human CD56 antibody (diluted 1:125) and the matched negative control (anti-mouse IgG_1) (diluted 1:500). Slides were washed twice for 5 minutes in TBS. The secondary antibody, biotinylated horse anti-mouse (1:200 dilution) was added to the slides and left at room temperature for 30 minutes, followed by two 5 minute washes in TBS. The tertiary complex was made up in TBS 30 minutes prior to use from an ABC Elite kit (Vector). It was added to the slides and left for 30 minutes at room temperature. Two 5 minute washes in TBS were carried out prior to addition of DAB (Dako). Once staining had developed in the positive control, slides were washed in dH_2O and mounted following haematoxylin counterstain and dehydration stages. The CD56 immunostaining results are represented in figure 6.5.

Figure 6.5

CD56 Immunostaining in decidual cells: positive and negative column fractions. Cells from the negative column fraction (n = 2) (**a** and **b**). Cells from the positive selection column fraction (n = 2) (**c** and **d**). Negative control for the positive selection column fraction (**e**). Scale bar represents 50μm.


a
b
c
d
e

6.2.6 Cell Culture

The purified decidual (n = 5) and PB NK cell populations (n = 5) were seeded in 12-well plates at a concentration of $1.4x10^5$ cells/ml (see figure 6.6). Treatment regimes were added in duplicate wells as in table 6.1.

Table 6.1

Treatment of uNK and PB NK cells with PGE_2		
Treatment	**Concentration**	**Incubation time**
Indomethacin (Control)	5μM	24 hours
IL-15 + indomethacin	5ng/ml + 5μM	
PGE_2 + indomethacin	10^{-6}M + 5μM	
IL-15 + PGE_2 + indomethacin	5ng/ml + 10^{-6}M + 5μM	

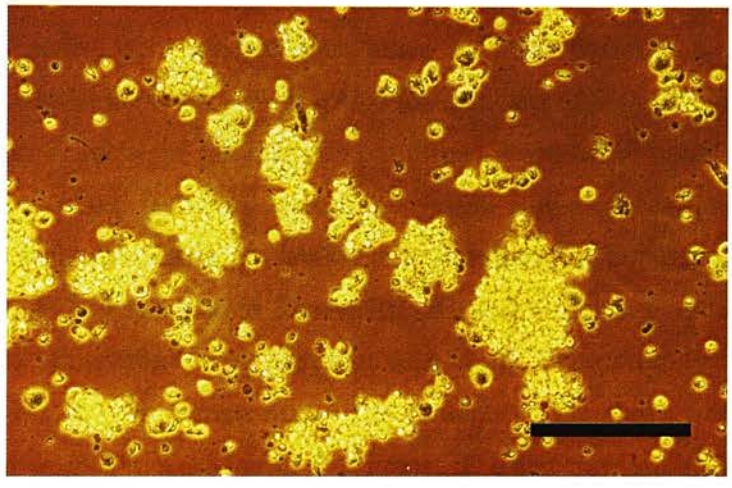


Figure 6.6

uNK cells after 24 hours in culture media. Scale bar represents 150μm.

6.2.7 IL-10 ELISA

Media collected from the uNK cell (n = 4) and PB NK cell (n = 5) culture experiments after 24 hours and was assayed for IL-10 using a two-site sandwich ELISA (section 2.6.1). Plates were initially coated with human IL-10 capture antibody (Pharmingen, Beckton Dickinson Company, Oxford, UK) at 2μg/ml diluted in phosphate buffered saline. 100μl was added to each well. Plates were left for 1 hour before being washed twice in water. To each well, 400μl of Blocking and protecting medium was added and left at room temperature for 60 minutes. The Blocking and protecting medium was aspirated and plates were washed four times in wash buffer. A standard curve (Pharmingen) was added to each plate with a top standard of 500pg/ml made up in PBS with 10% FCS. A 1:2 serial dilution was performed down to 7.8 pg/ml to create seven standards in total. Four replicates of a quality control with a concentration of 100pg/ml were added to enable intra-assay variability to be monitored. To measure NSB, 100μl of PBS with 10% FCS was added to four wells. All test samples were run undiluted, in duplicate wells with 100μl being added to each well. Plates were placed on a plate shaker for 1 hour at room temperature. The contents of the plates were aspirated and they were then washed in wash buffer four times. The detection antibody was diluted in PBS plus 10% FCS to 1.3μg/ml and 100μl added to all wells. Plates were left for 1 hour at room temperature on a plate shaker and then washed four times in wash buffer. The streptavidin peroxidase was added at 0.125U/ml, each well receiving 100μl. Plates were sealed and placed on a plate-shaker for 20 minutes at room temperature followed by the standard washing procedure. The substrate was mixed (see section 2.6.1) and 200μl transferred to each well. Each plate was allowed 20 minutes to develop and then read on a Multiscan Ex plate reader (Labsystems) using a filter with a 450nM absorbance value. The computer program Assay Zap was used to analyse the results and construct a standard curve against which the samples could be compared. The intra-assay variation was calculated from 8 replicates of the same standard on a plate and found to be 15.4%. The inter-assay variation was calculated across 4 separate assay plates and was determined to be 18.3%.

6.2.8 RNA Extraction and Q RT-PCR

The RNA was extracted and cDNA prepared from the cell culture experiments. IFN-γ, CD40L, IL-10 and EP_2 and EP_4 receptor mRNA levels were measured by Q RT-PCR as described in section 2.5.

6.2.9 Statistical Analysis

Q RT-PCR and ELISA data were analysed by the methods described previously in section 2.8.

6.3 Results

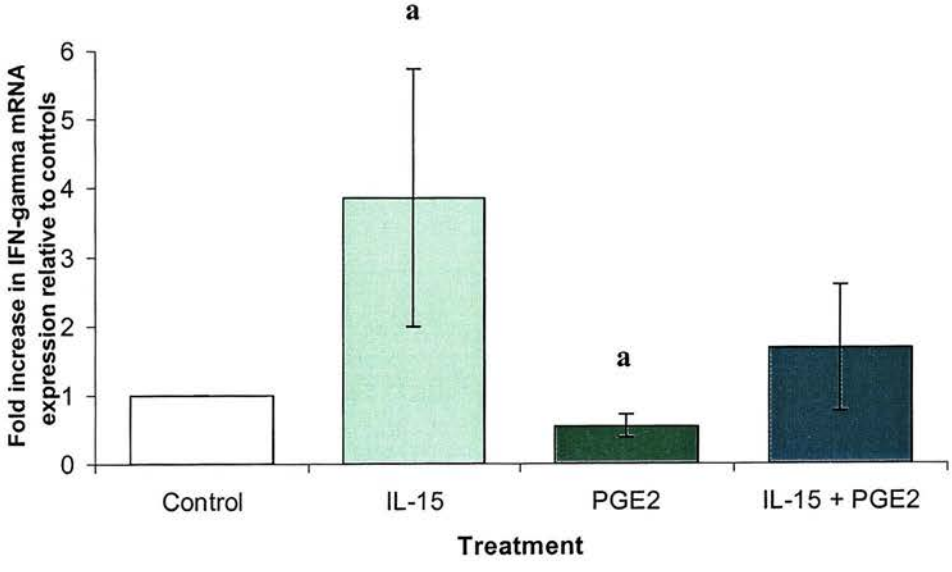
6.3.1 IFN-γ mRNA Expression in uNK Cells

The results in figure 6.7 show that following IL-15 treatment for 24 hours IFN-γ mRNA levels had increased almost 4-fold in relation to controls and 4-fold compared with the PGE_2 treatment group ($p < 0.04$). When PGE_2 was added alongside IL-15 the rise in mRNA levels with IL-15 treatment alone was reduced to just under a 2-fold increase. The level of IFN-γ mRNA expression was lower in the PGE_2 treated cells than in the controls.

Figure 6.7

The relative IFN-γ mRNA expression levels in uNK cells following a 24 hour incubation with IL-15, PGE_2 and IL-15 plus PGE_2. Same letters denote significant difference. **a** $p < 0.04$.

Figure 6.7


a
a
Fold increase in IFN-gamma mRNA expression relative to controls
0
1
2
3
4
5
6
Control
IL-15
PGE2
IL-15 + PGE2
Treatment

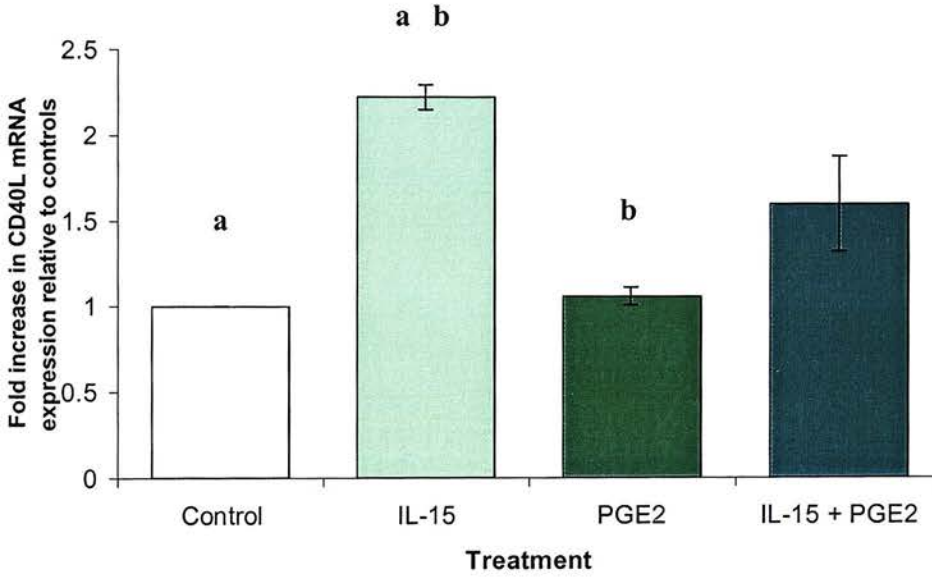
6.3.2 CD40-Ligand mRNA Expression in uNK Cells

The relative levels of CD40L mRNA was over 2-fold greater in the IL-15-treated cells than in the controls ($p < 0.004$) (figure 6.8). When added alone, PGE_2 had no effect on expression levels inducing comparable levels to controls. However, when PGE_2 was added in combination with IL-15 it appeared to inhibit the stimulatory effects of IL-15 modestly although this was not a significant reduction.

Figure 6.8

The relative CD40L mRNA expression levels in uNK cells following a 24 hour incubation with IL-15, PGE_2 and IL-15 plus PGE_2. Same letters denote significant difference. **a** $p < 0.004$ and **b** $p < 0.006$.

Figure 6.8


a b
a
b
2.5
2
1.5
1
0.5
0
Fold increase in CD40L mRNA expression relative to controls
Control
IL-15
PGE2
IL-15 + PGE2
Treatment

6.2.3 EP_2 and EP_4 Receptor mRNA Expression in uNK Cells

The uNK cells were expressing both the EP_2 and EP_4 receptor according to mRNA levels. No change in the EP_2 receptor was observed following IL-15 and PGE_2 treatments according to the results in figure 6.9. The mRNA expression levels of the EP_4 receptor increased 3-fold relative to controls following IL-15 treatment ($p < 0.01$) and PGE_2 plus IL-15 triggered a 4-fold rise in the EP_4 receptor ($p < 0.0006$) (figure 6.10). PGE_2 added alone had no effect on EP_4 receptor expression.

Figure 6.9

The relative EP_2 receptor mRNA expression levels in uNK cells following a 24 hour incubation with IL-15, PGE_2 and IL-15 plus PGE_2.

Figure 6.9

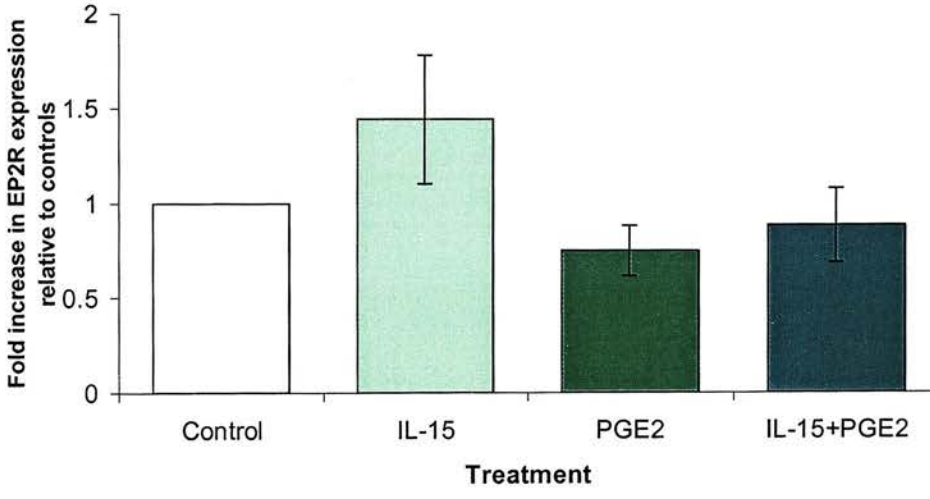
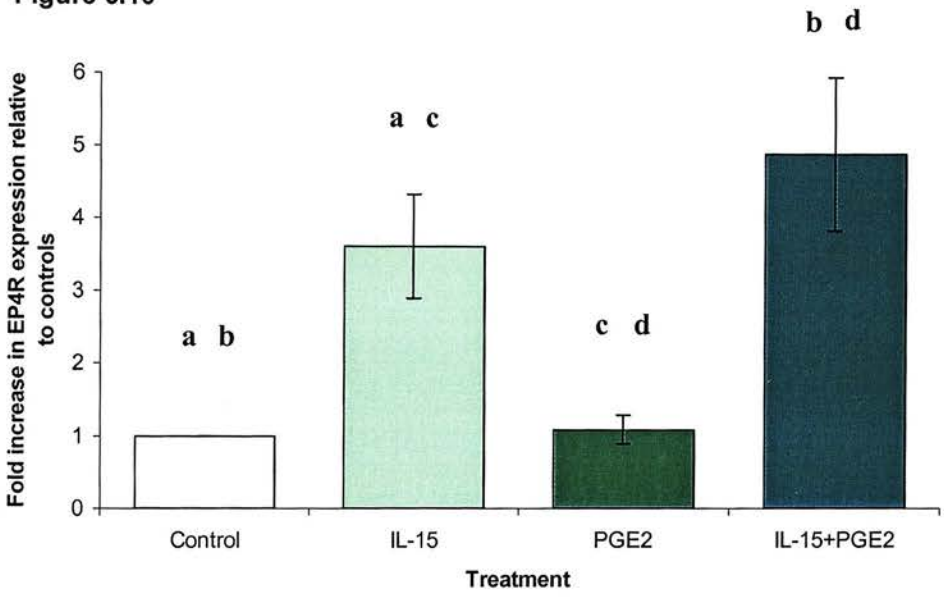

Fold increase in EP2R expression relative to controls
2
1.5
1
0.5
0
Control
IL-15
PGE2
IL-15+PGE2
Treatment

Figure 6.10

The relative EP_4 receptor mRNA expression levels in uNK cells following a 24 hour incubation with IL-15, PGE_2 and IL-15 plus PGE_2. Same letters denote significant difference. **a** $p < 0.01$; **b** $p < 0.0006$; **c** $p < 0.014$ and **d** $p < 0.0007$.

Figure 6.10


b d
a c
a b
c d
Fold increase in EP4R expression relative to controls
0
1
2
3
4
5
6
Control
IL-15
PGE2
IL-15+PGE2
Treatment

6.3.4 IL-10 mRNA Expression and Release by uNK Cells

uNK cells treated with PGE_2 stimulated a 3.5-fold increase in IL-10 mRNA levels compared with controls ($p < 0.0004$) (figure 6.11). This trend was consistent with IL-10 release from uNK cells although the exact levels detected were variable between the 4 samples (figure 6.12 a-d) and the fold changes in protein levels are illustrated in figure 6.13. IL-15 had no effect on IL-10 mRNA levels relative to controls and this was consistent with the IL-10 ELISA data (figure 6.13).

Figure 6.11

The relative IL-10 mRNA expression levels in uNK cells following a 24 hour incubation with IL-15, PGE_2 and IL-15 plus PGE_2. Same letters denote significant difference. **a** $p < 0.0004$; **b** $p < 0.005$; **c** $p < 0.0003$ and **d** $p < 0.0032$.

Figure 6.11

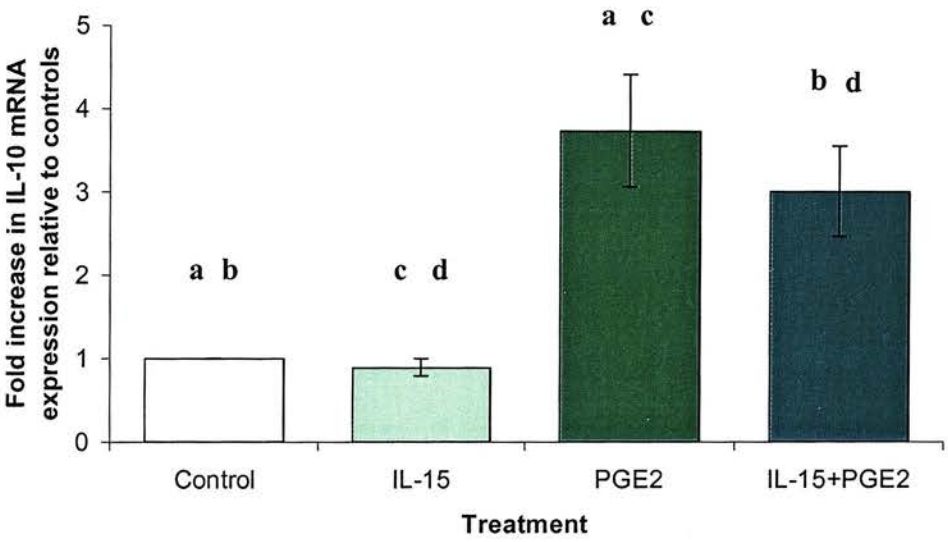

Fold increase in IL-10 mRNA expression relative to controls
5
4
3
2
1
0
a b
c d
a c
b d
Control
IL-15
PGE2
IL-15+PGE2
Treatment

Figure 6.12 a-d

IL-10 release in pg/ml over 24 hours from uNK cells in the four separate cultures as determined by ELISA. The lower detection limit of the ELISA was 7.8pg/ml.

Figure 6.12a

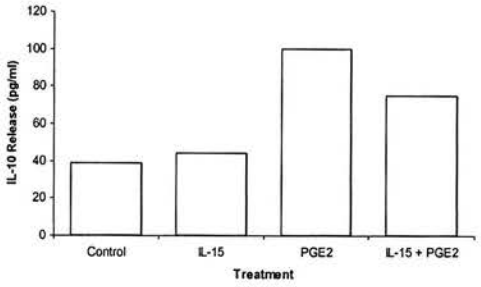


Figure 6.12b

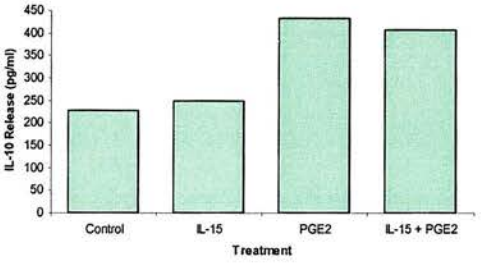


Figure 6.12c

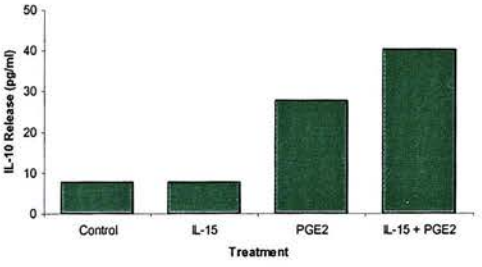


Figure 6.12d

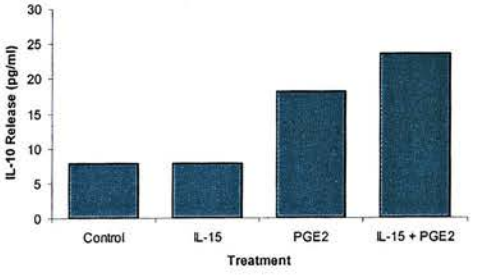
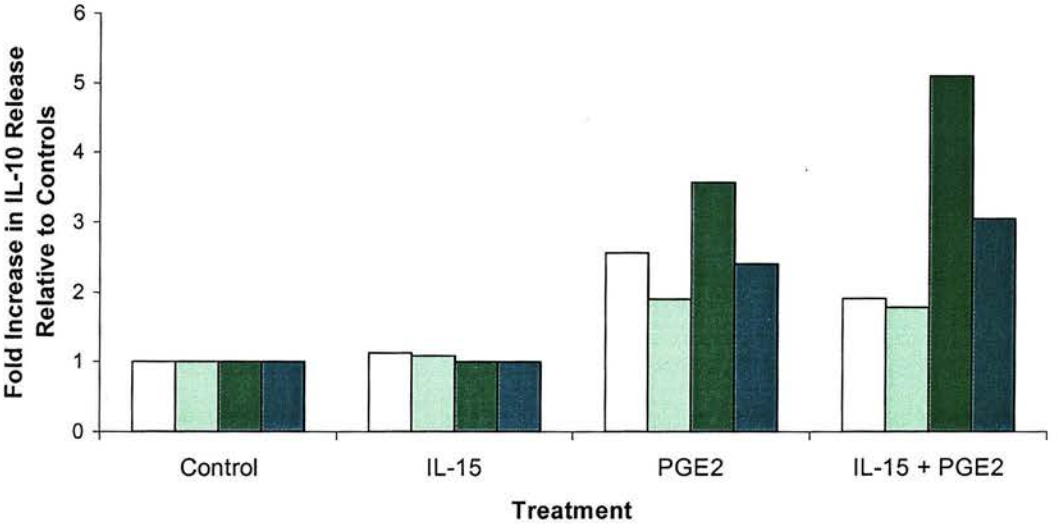


Figure 6.13

Summary of the mean values of IL-10 released from uNK cells over 24 hours (from the data in figure 6.12 a-d) and represented as fold change in secretion levels relative to controls.

Figure 6.13


Fold Increase in IL-10 Release Relative to Controls
6
5
4
3
2
1
0
Control
IL-15
PGE2
IL-15 + PGE2
Treatment

6.3.5 IL-10 mRNA Expression and Release by PB NK Cells

IL-10 mRNA levels were increased approximately 7-fold in relation to controls following a 24 hour treatment with PGE_2, although this was not significant ($p < 0.067$) (figure 6.14). IL-15 had no appreciable effect on IL-10 mRNA levels. The ELISA data (figure 6.15) was, however, variable and inconsistent with the mRNA levels. There were no significant differences in IL-10 protein levels relative to controls with either PGE_2 or IL-15 treatment.

Figure 6.14

The relative IL-10 mRNA expression levels in PB NK cells following a 24 hour incubation with IL-15, PGE_2 and IL-15 plus PGE_2.

Figure 6.14

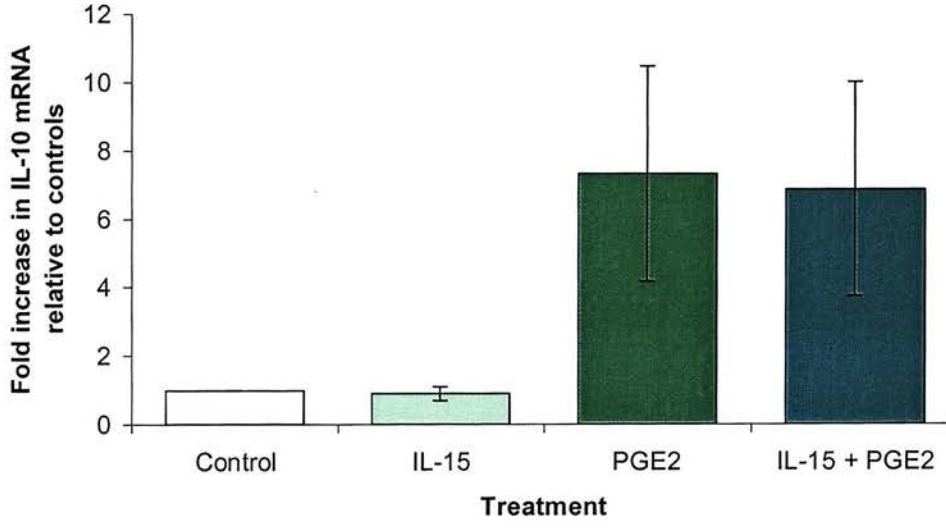
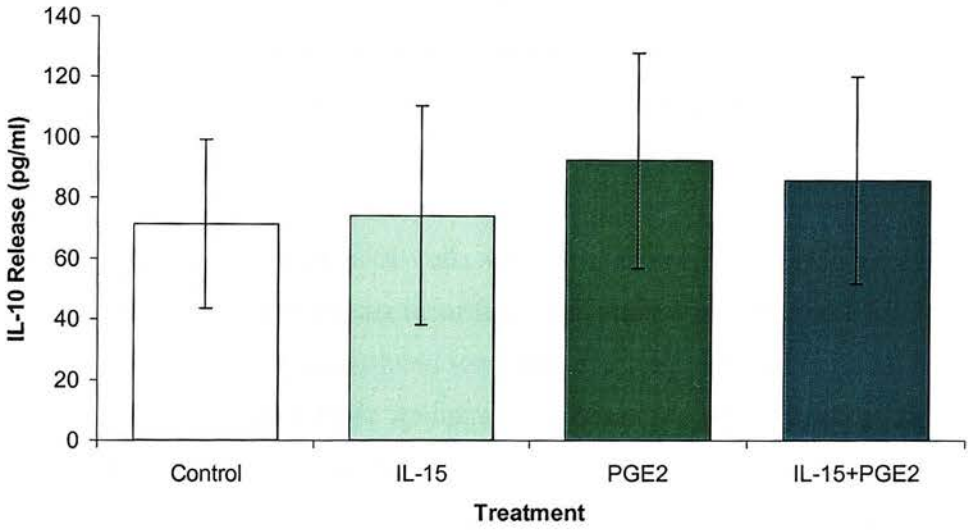

Fold increase in IL-10 mRNA relative to controls
12
10
8
6
4
2
0
Control
IL-15
PGE2
IL-15 + PGE2
Treatment

Figure 6.15

IL-10 release in pg/ml over 24 hours from PB NK cells incubated with IL-15, PGE_2 and IL-15 plus PGE_2. The lower detection limit of the ELISA was 7.8pg/ml.

Figure 6.15


140
120
100
80
60
40
20
0
IL-10 Release (pg/ml)
Control
IL-15
PGE2
IL-15+PGE2
Treatment

6.4 Discussion

The results detailed in this chapter have shown the ability of IL-15 to stimulate a significant rise in IFN-γ mRNA levels in uNK cells *in vitro* compared with PGE_2-treated uNK cells. In addition, IL-15 treatment gave rise to a significant increase in CD40L mRNA levels in comparison to controls and equivalent PGE_2-treated cultures. IL-15 had no effect on the EP_2 receptor expression levels. However, IL-15 induced a significant rise in the EP_4 receptor mRNA levels relative to controls and uNK cultures treated with PGE_2. PGE_2 stimulated a significant rise in IL-10 mRNA levels in uNK cells compared with controls and the same trend was apparent when IL-10 secretion was assayed although due to variability of IL-10 levels secreted, this was not significant. IL-15 had no effect on IL-10 mRNA expression. In the PB NK cell cultures, PGE_2 appeared to be having a stimulatory effect on IL-10 mRNA levels and in all cultures PGE_2 stimulated a rise in IL-10 expression. However, due to the variability between samples this was not significant.

The preparations of separated uNK cells were on average 75% CD56 positive. The remaining 25% of the cells were not identified. It is unlikely that they are ESCs because they are adherent and the cells cultured were non-adherent. The other possibilty is that they are macrophages since these immune cells constitute approximately 20% of the decidual leukocyte population (Loke *et al.,* 1995). These cells may be contributing to some extent to the results attained in this chapter since they are a potential source of IFN-γ (Gessani and Belardelli, 1998). This should be taken into account when considering the results although it has been confirmed that the predominant cell in the cultures is the uNK cell. The viability of the cultured cells was not examined and it may be that some of these cells may have been dead. Culture time was kept to a minimum of only 24 hours and evidence of alterations in cytokine production after different treatments implies the presence of living cells. However, in future studies it may be important to confirm the viabiltiy of the cells.

The role of uNK cells in reproductive functions has been proposed and explored in several review papers and have been implicated in decidualisation, implantation and menstruation (King *et al.*, 1990; King, 2000; Loke *et al.*, 2000; Croy *et al.*, 2002). Animal studies have demonstrated that mice lacking uNK cells are fertile and become pregnant without complications (Greenwood *et al.*, 2000; Miyazaki *et al.*, 2002). They do, however, experience defective decidualisation and it is possible that this would be of greater consequence in humans where decidualisation is a pre-requisite for successful embryo implantation. Additionally, inadequate conversion of the decidual vessels was observed in these mice. One strain of uNK cell-deficient mice, tgε26, exhibit reduced placental size and the offspring have reduced birth weight (Greenwood *et al.*, 2000). The deficient decidualisation and reduced placental growth could potentially have effects on fetal programming and thus lead to systemic effects later in life as with increased cardiovascular risk factors in adults with dysfunctional growth *in utero* (Barker, 1997a). NK cells have been postulated to have importance in early pregnancy maintenance in humans. High activity and raised levels of PB NK cells are associated with recurrent spontaneous abortion (RSA) of an unknown aetiology (Ntrivalas *et al.*, 2001; Yamada *et al.*, 2001). When women with received massive intravenous immunoglobulin (MIVIg) treatment, a decrease in activity and number of $CD56^{+}$ PB NK cells was observed followed by successful pregnancies (Morikawa *et al.*, 2001). In the uterus, a significant rise in CD56 positive cells is apparent in women suffering from RSA (Quenby *et al.*, 1999) suggesting raised levels are either detrimental to early pregnancy or a failed attempt to maintain pregnancy. In contrast, lowered cytotoxicity of PB and uNK cells has also been implicated in endometriosis as discussed in Somigliana *et al* 1999 (Somigliana *et al.*, 1999). The conclusions drawn from these studies should, however, be viewed with caution. The uNK cells represent a distinct sub-class of NK cells and whether peripheral NK cells have any influence on uNK cells and pregnancy outcomes has yet to be established. In other studies the number of uNK cells was assessed in decidua following spontaneous miscarriage in comparison to normal decidua (Vassiliadou and Bulmer, 1996; Kodama *et al.*, 1998). Both studies

reported a rise in uNK cells in the miscarriage decidua versus controls although these results may actually represent post-miscarriage inflammatory alterations.

PGE_2 has been proposed to inhibit the anti-trophoblastic action of decidual leukocytes via inhibition of the IL-2 receptor (Parhar *et al.*, 1989). In studies on mice NK cells activated with IL-2, PGE_2 supplementation decreased their lytic activity, prevented proliferation and increased the size and granularity of the cells (Linnemeyer *et al.*, 1993). They proposed that the PGE_2 could be differentiating NK cells at the implantation site via the same mechanism and would allow successful implantation. The results in this chapter have shown the ability of PGE_2 to increase IL-10 levels in uNK cells *in vitro*. PGE_2 has been implicated in inhibition of IL-15-mediated IFN-γ production in human PB NK cells via the common γ-chain of the IL-15 receptor. PGE_2 also acted to decrease the cytotoxicity of IL-15 activated NK cells (Joshi *et al.*, 2001). The results from this study and in this chapter suggest that PGE_2 is having the effect of inhibiting the Th-1 response and favouring the Th-2 pathway. In a study of patients undergoing IVF, serum IL-10 levels were raised in the period following successful implantation, thus implying IL-10 in sustaining normal pregnancy (Wu *et al.*, 2001). However, the simplistic theory that Th-1 cytokines are detrimental to pregnancy and that a Th-2 response is critical to pregnancy maintenance (Wegmann *et al.*, 1993) has been questioned and it appears to be far more complex. Work on mice has demonstrated that IL-10 and IL-4 from either maternal or feto-placental sources are not essential to pregnancies as was previously thought (Svensson *et al.*, 2001). However, this study did not examine if there were any compromising effects on fetal and post-natal development but does suggest that the classical Th-1/Th-2 hypothesis is too basic. It is also possible that other cytokines may be compensating for the deficiency in IL-4 and IL-10 which further complicates the situation. In a detailed study the immuno-expression of a wide range of cytokines was assessed in the uterus, within the peri-implantation embryo and in decidual and placental tissues in mice (Chaouat *et al.*, 2002). They demonstrated that Th-1 cytokines, such as IL-12, are present at the implantation site in normal pregnancies. If a similar finding is confirmed within humans this implies that the Th-1/Th-2 theory is

an over-simplification and it is probable that the situation is complex with fine-tuning necessary.

The results in this chapter noted variability in the levels of IL-10 secreted from the uNK cells. Previously, uNK cells have been demonstrated to express both IL-10 and the IL-10 receptor mRNA. In addition, the proinflammatory cytokines, IL-2 and IL-12, were shown to increase IL-10 production (Vigano *et al.*, 2001). In a study of IL-10 and IFN-γ levels in decidual and blood mononuclear cells from elective first trimester terminations, levels were found to be variable between patients. However, in women with relatively high IL-10 levels the IFN-γ levels were also raised (Ekerfelt *et al.*, 2002). This implies that it is the ratio of these two cytokines that is important to the stability of early pregnancy. In IL-10 -/- mice placental deformities are apparent and it is possible that IL-10 has a role in modifying placental growth and structure and therefore fetal programming (Roberts *et al.*, 2003). In women with missed abortions, decreased levels of IL-10 immunostaining was observed in seven out of ten patients compared with elective terminations. The levels of $CD56^+$ cells were comparable between the groups as was IFN-γ staining intensity, suggesting the importance of the cytokine profile in pregnancy maintenance (Plevyak *et al.*, 2002). When post-miscarriage decidua is used in a study it is important to be aware that inflammatory alterations and necrosis may be factors affecting the results obtained. Abnormally raised levels of IL-10 can also be detrimental outside the decidua to normal pregnancy functions. Raised IL-10 levels in mid-trimester amniotic fluid are associated with small for gestational age (SGA) babies in first pregnancies (Heyborne *et al.*, 1994).

The results in chapter 5 have illustrated the expression of IFN-γ mRNA across the menstrual cycle. Immunohistological studies have localised IFN-γ to the lymphoid aggregates (Stewart *et al.*, 1992a) although this is in contrast to the results of another study that identifies polymorphonuclear neutrophils and intraepithelial lymphocytes as the source of IFN-γ (Yeaman *et al.*, 1998). First trimester decidual NK cells have been

shown to express IFN-γ in addition to LIF and GMCSF (Jokhi *et al.*, 1994a). In this chapter the Th-1 cytokine, IL-15, has been shown to raise significantly uNK cell IFN-γ mRNA levels. In chapter 5 IFN-γ was shown to be essential to IL-15 secretion by ESCs. This offers a potential mechanism by which ESCs and uNK cells may be functionally linked (figure 6.16). Regulation of IFN-γ levels appears to be critical to normal endometrial function since women with endometriosis express raised levels of this cytokine compared with healthy controls (Klein *et al.*, 1993). Mice studies offer a further potential role for IFN-γ in uterine function. IFN-γ -/- and IFN-γ receptor -/- mice show abnormal granulation of uNK cells at the implantation site and abnormal decidual transformation of the decidual arteries (Ashkar *et al.*, 1999). This has been shown to be uNK-derived IFN-γ by further experiments involving bone marrow grafts (Ashkar *et al.*, 2000).

The expression of CD40L has not been studied with regard to uNK cells previously. In this chapter CD40L levels were raised by IL-15 treatment. ESCs express CD40, as shown by immunohistochemistry and cell culture experiments (King *et al.*, 2001). ESCs and uNK cells are believed to exist in close proximity to one another (King, 2000) and this provides the contact needed for the ligand to bind CD40 on ESCs providing another link between these distinct cell types.

In summary, the cytokine IL-15 is able to raise expression of CD40L mRNA and also IFN-γ levels in uNK cells thus providing possible links with ESC function. In contrast, PGE_2 is acting to raise IL-10 expression, thus maintaining the Th-1/Th-2 balance essential for a normal pregnancy. It is possible that PGE_2 is acting via its EP_2 receptor upon rising levels following IL-15 stimulation but this is yet to be confirmed.

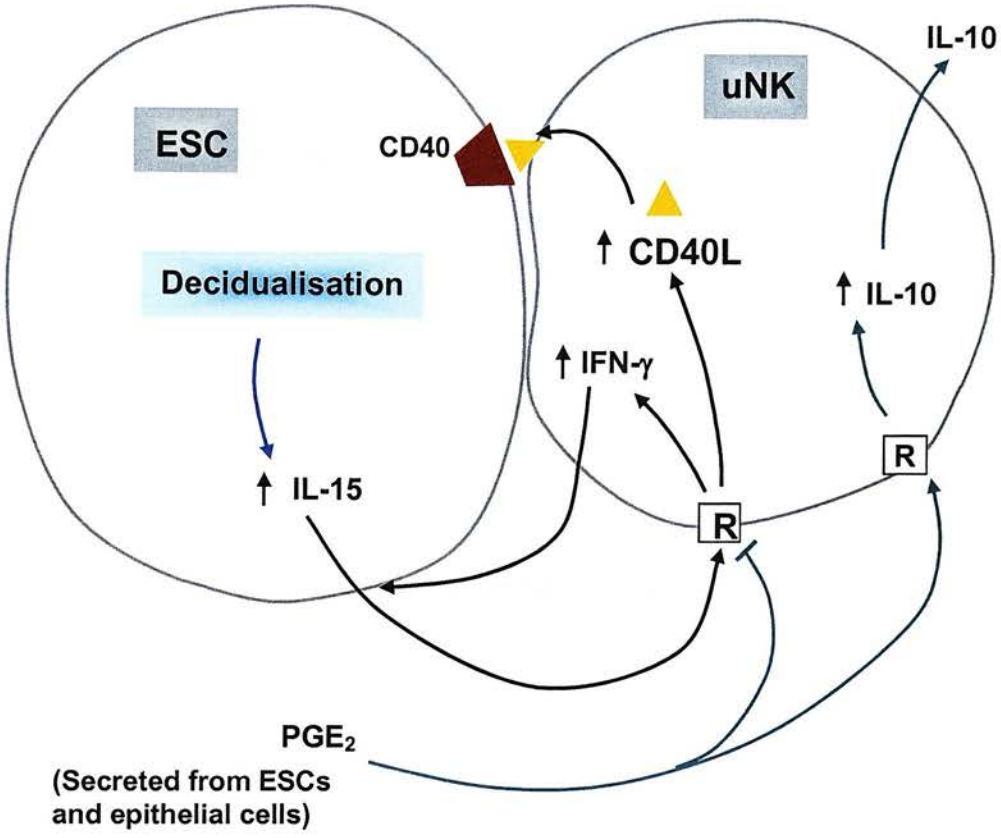


Figure 6.16

Illustration of how the actions of IL-15 and PGE_2 may be modifying uNK cell function. PGE_2 appears to have immunosuppressive actions via raising IL-10 levels and inhibiting the IL-15 receptor. IL-15 is stimulating IFN-γ and CD40L expression and in turn, these may be "feeding back" and acting on the ESC.

7. General Discussion

7.1 Synopsis of Results

These studies have demonstrated some of the unexpected changes that are occurring in ESCs during decidualisation. These may represent important modulations that are critical in regulating both the progression of decidualisation and the functions of other endometrial cell types such as the uNK cell. Here, the cytokine IL-15 has been linked to both ESC decidualisation and to regulating the functions of uNK cells with regard to their pro-inflammatory pathway response. In addition, the actions of PGE_2 and cAMP on aspects of the prostaglandin cascade have been analysed. The direct influence of PGE_2 on uNK cells has also been studied.

The effects of cAMP at various stages of the prostaglandin pathway during decidualisation in ESCs have been demonstrated here. mRNA levels of both COX enzymes are raised in response to cAMP, implying that this may result in an increase in PG production in these cells. PGE_2 has been shown to have a role in decidualisation (Frank *et al.*, 1994) and this present study has also shown that cAMP induces a rise in the expression of the EP_2 receptor which would provide a potential target for PGE_2 to act on, to allow progression of decidualisation. However, this rise in cAMP also raises mRNA levels of the phosphodiesterase enzymes, PDE3a and PDE4b, which are responsible for the breakdown of cAMP. This may be a self-regulating mechanism to prevent excessive levels of intracellular cAMP building up. In addition, relaxin may also target the pathway at the phosphodiesterase level to inhibit the action of phosphodiesterases and prevent cAMP destruction (Bartsch *et al.*, 2001). One of the major products of this pathway, PGE_2, has been shown herein to raise both its own receptor, EP_4, and IL-10 mRNA levels in uNK cells and may be important in maintaining the balance of Th-1 and Th-2 cytokine levels in the endometrium with regard to a successful pregnancy (Wegmann *et al.*, 1993). However, this production of IL-10 is unlikely to effect the production of IFN-γ by uNK cells (Vigano *et al.*, 2001).

As detailed previously, IL-15 mRNA levels increase in human ESC cultures after IFN-γ and PGE_2 treatment. In addition, the presence of IFN-γ was found to be essential to induce secretion of IL-15 protein from these cells in the presence of decidualising stimuli. The results of this study implicate both cAMP and IFN-γ as having central roles in IL-15 production. IL-15 is tightly regulated at the post-transcriptional level (Tagaya *et al.*, 1996) and this study has demonstrated that different factors are required to allow the optimal increase in mRNA expression in addition to facilitation of protein release, thus representing divergent and complex control mechanisms. It has previously been hypothesised that cells retain a pool of translationally inactive IL-15 mRNA to allow a rapid and effective response to stimuli when required to activate NK cells (Tagaya *et al.*, 1996). This has implications in both an immune response in the endometrium and for the reproductive functions of uNK cells.

It has been shown that IL-15 can induce a rise in both IFN-γ and CD40L mRNA in uNK cells *in vitro*. Additionally, PGE_2 acts on uNK cells to raise mRNA levels of the EP_4 receptor and IL-10. These results will be discussed in more detail in section 7.3 below.

IL-15 mRNA in ESCs rises in concordance with increasing levels of the decidualisation markers, prolactin and IGFBP-1, during ESC decidualisation *in vitro*. In a simulation of early pregnancy, IL-15 protein levels *in vivo* were found to be raised in relation to the luteal phase in both the glandular and stromal compartments. This is not unsurprising in the stroma since it will have been transformed into its decidualised state. However, the increase of IL-15 immunostaining intensity in the epithelial cells is less accountable. This study examined two human epithelial cell lines and confirmed that the factors regulating IL-15 mRNA expression in the primary ESCs were not the same. This distinct control over IL-15 in the two separate endometrial compartments may suggest separate functions of this cytokine. The rise in IL-15 in the simulation of early pregnancy group implies progesterone-directed control over its expression. If this is the case, then it is likely that the ESC, due to its persistent PR-A expression (Wang *et al.*, 1998), will provide this link between progesterone and the epithelial IL-15 production.

7.2 The Relevance of IL-15 in Normal Endometrial Function

Endometriosis affects around 10% of women of reproductive age presenting with the symptoms of pelvic pain and infertility (Giudice *et al.*, 1998). Using a microarray it has been demonstrated that endometrium from women with endometriosis have lower levels of IL-15 compared with control endometrium (Kao *et al.*, 2003). This evidence implicates IL-15 in having an impact on fertility beyond the confinements of decidualisation. In contrast, elevated levels of uterine IL-15 expression are present in women suffering from recurrent spontaneous abortion compared with healthy controls (Chegini *et al.*, 2002). It appears that the balance of IL-15 levels within the endometrium can be critical to uterine normal function and early pregnancy maintenance. The significance of IL-15 in the secretory phase is implicated by raised levels of this cytokine (Carson *et al.*, 2002), and the α chain of the IL-15 receptor, in the mid secretory phase compared with the early secretory and proliferative phases and specifically during the implantation window (Kao *et al.*, 2002).

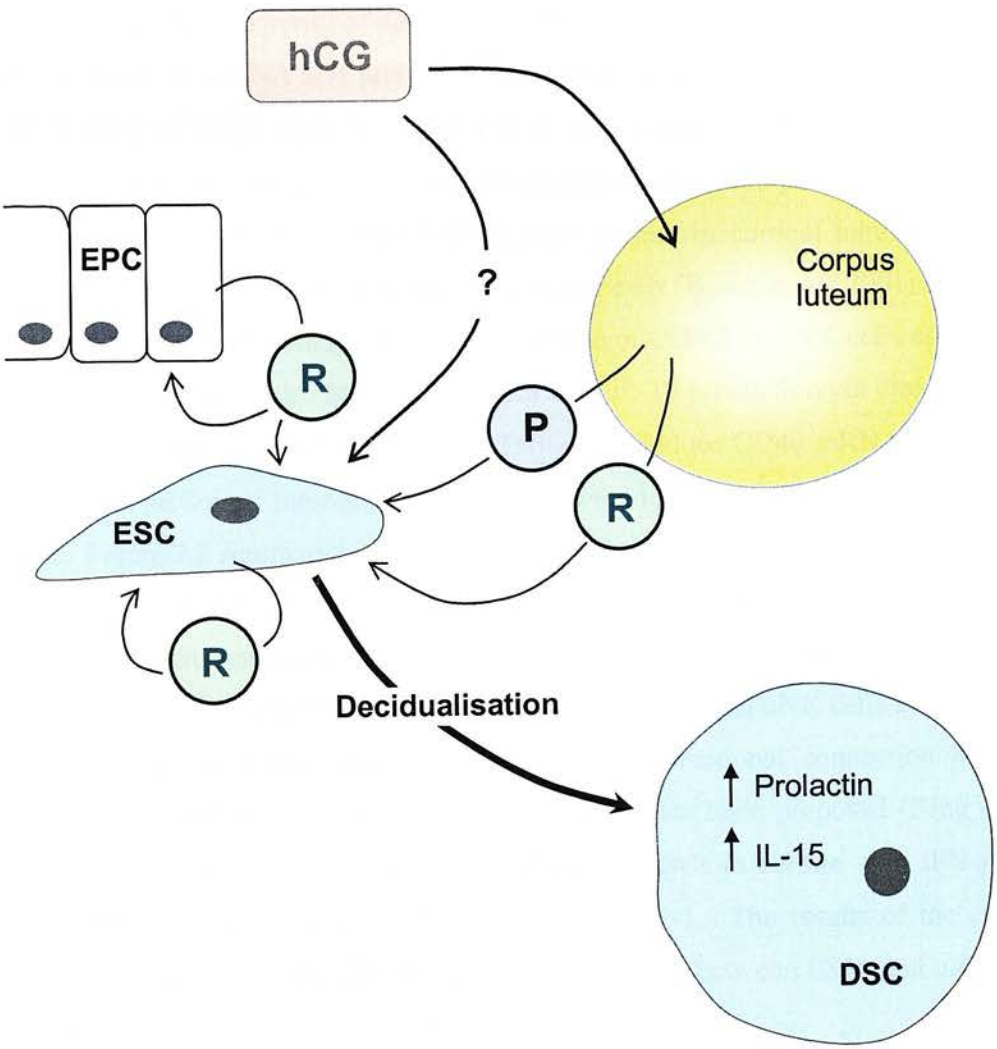
IL-15 levels of mRNA expression have been demonstrated here to rise alongside decidualisation of ESCs in culture. The implications of this may be of particular relevance to uNK cell functions since they proliferate upon IL-15 treatment *in vitro* (Verma *et al.*, 2000) and mice lacking the IL-2 receptor γ chain, utilised by IL-15, had no uNK cells (Miyazaki *et al.*, 2002). Since these mutated mice that lack uNK cells exhibit abnormal decidualisation and blood vessel transformation, it is possible that uNK cells have significant roles in implantation and placentation in humans. The mRNA level of IL-15 has been shown in chapter 5 to be raised in the mid secretory phase and this is likely to correlate with the necessity to regulate the functions of uNK cells at this critical stage in the reproductive cycle.

There are conflicting reports as to whether hCG receptors are present within the human endometrium. One study has demonstrated that treatment of ESCs *in vitro* with hCG is

unable to induce decidualisation (Kasahara *et al.*, 2001) whereas other studies contradict this, demonstrating a raise in intracellular cAMP levels (Chatterjee *et al.*, 1997) and PGE_2 levels (Han *et al.*, 1999) with hCG treatment. For hCG to be exerting these effects a functional receptor must be present on ESCs and immunohistochemistry has located hCG/LH receptors in the human endometrium in both glands and stroma with a rise being apparent in the secretory phase (Reshef *et al.*, 1990). Systemic hCG may therefore exert effects on the endometrium and this knowledge could have relevance to endometrial receptivity in women taking hCG as a fertility treatment to hyperstimulate the ovary (Fanchin *et al.*, 2001). The circulating hCG secreted by the trophoblast will potentially be having effects on the endometrium during the early stages of pregnancy. *In vivo* IL-15 immunostaining increases in women treated with hCG in a controlled simulation of early pregnancy study described herein. A rise in IL-15 levels in both the glandular and stromal compartments were observed. hCG is therefore likely to be producing these effects via the corpus luteum and possibly by direct action on the endometrial stroma. The effects of hCG on the corpus luteum will be to maintain progesterone production and stimulate the release of relaxin. These potential interactions are summarised in figure 7.1 below.

Figure 7.1

The potential mechanisms by which hCG may be influencing ESC decidualisation and IL-15 expression. hCG acts on the corpus luteum stimulating the secretion of progesterone (P) and relaxin (R) and may additionally be acting directly on the stromal cells. Relaxin is also produced within the endometrium by ESCs and by glandular epithelial cells (EPC). Relaxin may be having paracrine and autocrine effects on the ESCs alongside the action of progesterone inducing decidualisation in these cells. Upon decidualisation both prolactin and IL-15 levels increase in the ESCs thus forming decidualised stromal cells (DSC). In addition to the details of this figure, there are other factors that will be influencing decidualisation.

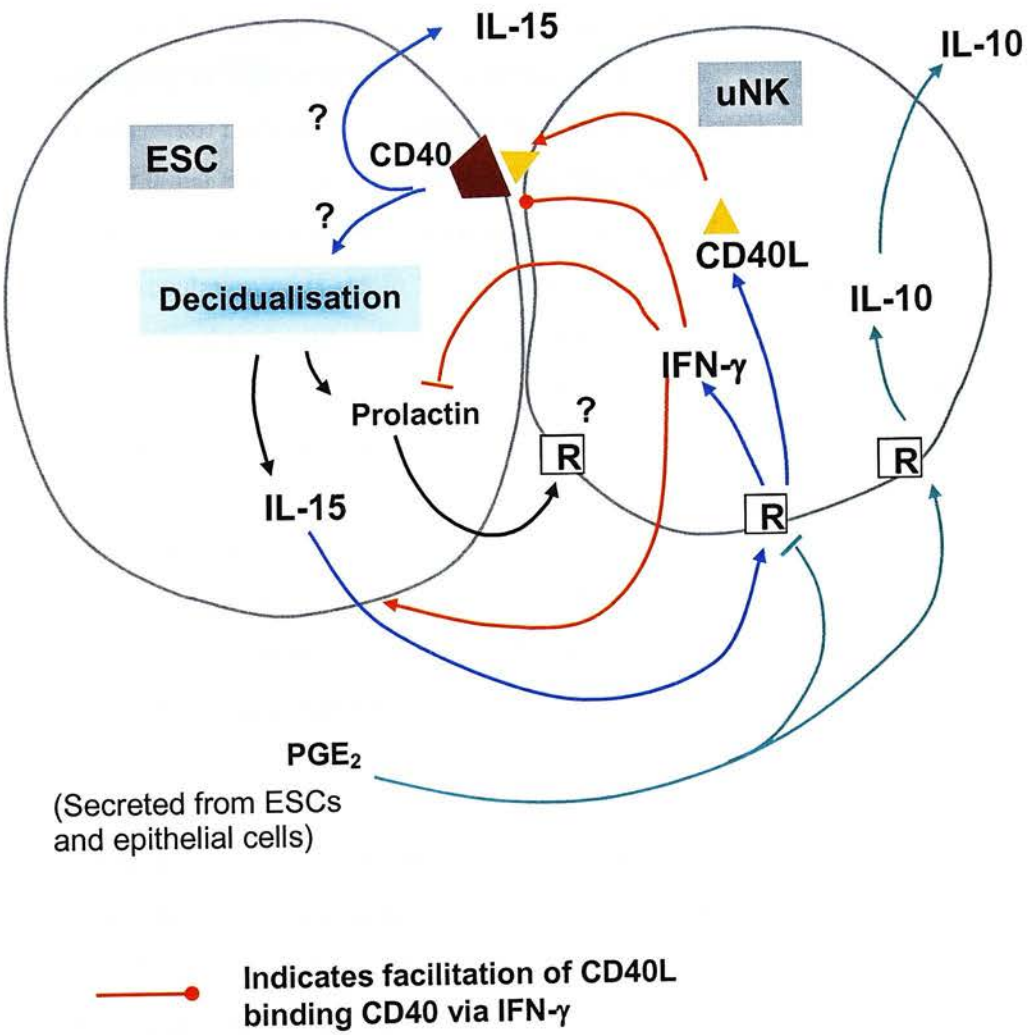

hCG
?
EPC
Corpus luteum
R
P
R
ESC
R
Decidualisation
Prolactin
IL-15
DSC

7.3 The Relationship Between the ESC and the uNK Cell

In a study of uNK cells it has been demonstrated here that IL-15 stimulates a rise in IFN-γ and CD40L mRNA levels. As discussed above, IFN-γ is able to trigger IL-15 release from ESCs and this may be a mechanism by which feedback occurs to ESCs. IFN-γ has been shown to inhibit activity of the prolactin promoter, therefore antagonising cAMP-mediated prolactin mRNA and protein in differentiating ESCs (Christian *et al.*, 2001). Since IL-15 production appears to be linked to decidualisation and thus prolactin production, IFN-γ may be providing additional regulation of this pathway at this level. Previous studies have shown that CD40 ligation on human cortical tubular epithelial cells (Weiler *et al.*, 2001) and peritoneal mesothelial cells (Basok *et al.*, 2001) stimulates IL-15 production. In this study the rising expression of CD40L in uNK cells upon IL-15 treatment may therefore also be contributing to ESC IL-15 production via direct cell-cell contact. It was also shown that IFN-γ and TNF-α can induce CD40 mRNA and protein expression in peritoneal mesothelial cells which would provide a ligation target for the CD40L. Figure 7.2 summarises this and hypothesises how these may be linked. Within the human endometrium immunoexpression of CD40 has been located to the perivascular and stromal cells (King *et al.*, 2001). The stromal cells are therefore targets for CD40L which the present study has shown to be a product of uNK cells *in vitro*. The role of the CD40-CD40L system in provision of a functional connection between resident endometrial cells and invading immune cells has been proposed (King *et al.*, 2001). They demonstrated that activation of human ESCs in culture with IFN-γ plus CD40L increased the secretion of IL-6, IL-8 and MCP-1. The results of the current study propose that the CD40-CD40L system may be a link between ESCs and uNK cells in the human endometrium.

Figure 7.2

A summary of the hypothesised interactions between ESCs and uNK cells and how control over the decidualisation pathway may be modified. The relevant receptors for the separate ligands are designated by (**R**). The blue arrows illustrate the potential downstream effects of IL-15 produced by the ESCs on uNK cells. The orange arrows represent the possible feedback mechanisms from uNK cells to ESCs in response to IL-15. Control mechanisms to regulate this pathway include PGE_2 and the arrows coloured green represent the potential actions of PGE_2. IFN-γ may be modulating ESC functions in a number of ways: by facilitating the secretion of IL-15 protein, inhibition of prolactin expression and therefore exerting control over decidualisation and by controlling uNK cell and ESC interactions via the CD40-CD40L system. CD40 may be having effects on IL-15 levels either via decidualisation or by a separate pathway to induce a direct influence over IL-15 production. Prolactin receptors have been demonstrated on uNK cells (Gubbay *et al.*, 2002) but the effects of prolactin on these cells has not yet been clarified.


IL-15
IL-10
uNK
ESC
?
CD40
?
CD40L
Decidualisation
IL-10
IFN-γ
Prolactin
?
R
R
R
IL-15
PGE2
(Secreted from ESCs
and epithelial cells)
Indicates facilitation of CD40L
binding CD40 via IFN-γ

7.4 Suggestions For Future Study

1. The current study has concentrated on IL-15 production in the ESC with regard to decidualisation. What is apparent is that IL-15 is also produced by the endometrial epithelial cells and the results here demonstrated a rise in IL-15 immunoexpression in conjunction with a simulation of early pregnancy. How IL-15 is being regulated is these epithelial cells and its function needs to be addressed to fully understand the impact of IL-15 on normal and pathological endometrial function. A dysregulation of IL-15 levels has been reported in pathological states such as endometriosis (Kao *et al.*, 2002) and women suffering from recurrent spontaneous abortion (Chegini *et al.*, 2002) and therefore the exact regulation of IL-15 in both stromal and epithelial compartments are likely to be critical to fertility. Experiments using primary endometrial epithelial cells should be conducted with treatments as described in chapter 5 to assess the regulation of IL-15 using ELISA and Q RT-PCR.

2. Prolactin is another potential regulator of uNK cell function and provides another connection to imply the intimate link between ESCs and uNK cells and their functions. The effects of prolactin on uNK cell cytokine production would be an interesting area to explore.

3. CD40L mRNA was shown to be both expressed by uNK cells and to be upregulated by IL-15 treatment *in vitro*. The implications of this on IL-15 production by ESCs should be explored in conjunction with IFN-γ to fully establish whether ESCs and uNK cells are linked via this mechanism. In particular, cultures of ESCs should be treated with IFN-γ and CD40L alone and in combination. The levels of CD40 mRNA expression should be assessed following these treatments by Q RT-PCR and protein production could be confirmed by immunohistochemistry. In addition, IL-15 levels and prolactin levels should also be examined. To further determine the CD40L expression by

uNK cells the cells could be analysed by FACS after being treated with IL-15 and PGE_2 as described in chapter 6. This would provide a further means to measure changes in CD40L expression on the uNK cells.

4. This study has begun to address how the PG pathway can be modified by cAMP. Exploration of these modifications will aid our understanding of decidualisation and its regulation at the intracellular level. It would be of value to explore further how PGE_2 secretion is regulated, possibly via the MRP4 transmembrane transporter. The secretion of PGE_2 by ESC cultures could be confirmed by ELISA and the levels of MRP4 expression examined by FACS and Q RT-PCR to determine whether a correlation is present.

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Appendix 1: Conference Proceedings

Dunn, C. L., Critchley, H.O.D. and Kelly, R.W. (2002 - February) IL-15 Regulation in Human Endometrial Stromal Cells. Munro Kerr, Glasgow, UK.

Dunn, C. L., Critchley, H.O.D. and Kelly, R.W. (2002) IL-15 Regulation in Human Endometrial Stromal Cells. NK Cells and Reproduction, Cambridge, UK.

Dunn, C. L., Critchley, H.O.D. and Kelly, R.W. (2003) IL-15 in the Decidualised Endometrium. Fertility 2003, Aberdeen, UK.

Appendix 2: Publications

Dunn, C. L., Critchley, H.O.D. and Kelly, R.W. (2002) IL-15 Regulation in Human Endometrial Stromal Cells. Journal of Clinical Endocrinology and Metabolism 87 (4): 1898-1901.

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IL-15 Regulation in Human Endometrial Stromal Cells

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Abstract A greater knowledge of IL-15 regulation within human endometrium is important in understanding key reproductive events and uterine Natural Killer cell function. In the present study, expression of IL-15 mRNA was shown to be up-regulated by both PGE_2 and IFN-γ in cultures of human endometrial stromal cells (ESC). Release of IL-15 protein was also shown to be under the control of PGE_2 and IFN-γ using an enzyme-linked immunosorbent assay for IL-15. In addition, 8-Bromo cAMP was able to increase IL-15 release from ESCs ($P < 0.0005$) implying the actions of PGE_2 may be via this second messenger. Addition of a progestin appeared to enhance these effects. Real-time quantitative PCR has demonstrated an up-regulation in IL-15 mRNA expression in the late secretory phase of the menstrual cycle ($P < 0.005$) and a progressive rise in IFN-γ expression throughout the secretory phase and into first trimester decidua. These results suggest that IL-15 regulation in the human endometrium is complex and that hormonal control may be indirect.

Cellular interaction in the uterus is critical in reproductive events, such as implantation, menstruation and placental formation. Natural Killer (NK) cells of the $CD56^{bright}$ CD16$^-$ phenotype are a major leukocyte population located in endometrium and first trimester decidua. Implied involvement of uterine NK cells at the time of endometrial decidualisation and in endometrial breakdown at menstruation (1) suggests a dynamic role for this cell type in uterine physiology. IL-15 has also been shown to be a potent stimulator of uterine NK cell proliferation (2). $CD56^{bright}$ NK cells produce immunoregulatory cytokines and chemokines such as GMCSF, IL-10, IL-13 and INF-γ. IL-15 is reported to be essential for type 2 cytokine production by these cells (3).

Immunohistochemistry has confirmed the localisation of IL-15 in human endometrium both perivascularly in secretory phase stromal cells and in glandular epithelial cells during the proliferative phase (4). Immunoreactivity for IL-15 persists in the stromal compartment in first trimester decidua (4). Previous studies have demonstrated the ability of progesterone to increase IL-15 expression and release by both endometrial stromal cells (ESCs) and decidual cells (4, 5) and a relationship between IL-15 and stromal cell decidualisation has been proposed (6). A combination of Medroxy progesterone acetate (MPA), a synthetic progestin, and the second messenger cAMP, has been shown to be an effective inducer of the decidualised phenotype (7, 8). This second messenger is involved in PGE_2 signal transduction through the EP2 and EP4 receptors (9) and PGE_2 causes differentiation of human endometrium (10).

Studies in mice show that IFN-γ is not only produced by uterine granular lymphocytes and is necessary for their development, but also is essential for appropriate blood vessel growth at implantation (11).

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In the present study the control of IL-15 expression and secretion by IFN-γ and PGE_2 in human ESCs is examined and expression of IL-15 and IFN-γ across the menstrual cycle determined using real-time quantitative PCR.

Materials and Methods

Tissue collection

Human endometrial specimens were collected from women of reproductive age (n=34) undergoing gynaecological procedures for benign indications. All women had regular menstrual cycles between 25-35 days and had not taken hormonal treatment within the 3 months prior to surgery. Specimens were dated according to the patient's last menstrual period (LMP) and were consistent with histological examination and circulating sex steroid concentrations.

Decidual (n=5) and trophoblast (n=4) specimens were obtained from women who had undergone surgical termination by vacuum aspiration, described previously (12), during the first trimester of pregnancy. The endometrial tissue was collected in sterile Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, Poole, Dorset, UK) and divided, whilst the decidual tissue was processed for messenger RNA (mRNA) extraction only: (i) homogenisation in tri-reagent to allow mRNA extraction; (ii) fixation in 10% neutral buffered formalin (NBF) overnight at 4°C followed by storage in 70% ethanol preceding wax embedding; and (iii) separation of cell types and culturing the endometrial stromal cells (ESCs).

Informed patient consent was obtained prior to tissue collection and ethics approval granted by Lothian Research Ethics Committee.

Cell culture

Endometrial specimens (n=11) were separated into epithelial and stromal cell populations by sedimentation. Specimens were washed in phosphate buffered saline and digested in collagenase for 80m at 37°C. An 18 gague needle was used to aid tissue breakdown. The cells were pelleted by centrifugation and re-suspended in 12ml RPMI 1640 and left for 5min to settle. The top 10ml were removed as the population of ESCs. Immunocytochemistry for vimentin and cytokeratin had been undertaken to verify the purity of cells separated by this technique. The ESCs were further cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (FCS) (Mycoplex; PAA, Teddington, UK), penicillin (50 μg/ml; Sigma), streptomycin (50 μg/ml; Sigma) and gentamycin (5 μg/ml; Sigma). Cells were cultured in $75cm^3$ culture flasks (Corning Incorporated, Corning, NY) for a minimum period of 5 days and allowed to reach confluence in the presence of estradiol (10^{-7} M), MPA (10^{-6} M) and basic fibroblast growth factor (bFGF) (5ng/ml).

Culture treatments and IL-15 ELISA

Once passaged, cells were seeded at a concentration of 1.4×10^5 cells/ml in 12-well plates (Corning Inc., Costar®) and incubated for 24h to allow cell adherence. Two separate ESC culture experiments were designed. In the first, ESC cultures (n=4) were divided into four treatment groups: (i) Control; (ii) PGE_2 (10^{-6}M); (iii) IFN-γ (25ng/ml); (iv) PGE_2+IFN-γ. Each well contained RPMI 1640 medium supplemented with 2% FCS and indomethacin (5μM). Extraction of mRNA was carried out at 4, 24, 48 and 72h and cDNA produced by reverse transcription. In the second culture experiment cells were divided into 7 treatment groups, each in quadruplicate wells: (i) Control; (ii) Control (without IFN-γ); (iii) MPA (10^{-6}M); (iv) 8-Bromo cAMP (0.1mg/ml) (Sigma); (v) MPA+8-Bromo cAMP; (vi) PGE_2; (vii) MPA+ PGE_2. All wells contained RPMI 1640 medium supplemented with 2% FCS and estradiol (10^{-7}M). After 24h, IFN-γ (25ng/ml) was added to all groups except (ii) and medium was harvested after a further 48h and stored at -20°C. Fresh medium and treatments were provided as before and again collected after 3 days of treatment. All medium samples were assayed for IL-15 in duplicate using a commercial kit (R&D Systems, Minneapolis, MN). The IL-15 ELISA inter-assay variation was calculated as a relative standard deviation and found to be 9.86% whilst intra-assay variation was 6.68%.

Real Time Quantitative PCR (RT-Q-PCR)

Real-time quantitative PCR was used to examine IL-15 and IFN-γ expression in human endometrial samples collected across the menstrual cycle, in first trimester decidua and in trophoblast (IL-15 only). IL-15 expression in cultured ESCs was also measured. Primers and probes for IL-15 and IFN-γ were designed using the PRIMER express program (PE Biosystem). Primers and probe for IL-15 were initially optimised and the linearity of the results validated via serial dilution of a cDNA pool. Calculation of the within assay variation was measured by amplification of six cDNA replicates. The PCR reactions were run on ABI Prism 7700 in duplicate and the same positive control was present in all runs to allow comparison. To determine genomic DNA contamination the β-actin signal was measured in all mRNA samples used in detection of IL-15/IFN-γ across the cycle and in representative control groups in the culture experiments. Samples were excluded from analysis if their β-actin signal fell below the arbitrary level of 27 cycles. The variability in the quantity of total mRNA in samples was measured by determining the relative standard deviation between 18-S values in the PCR results. This was found to be 1.77% in a single run, selected at random, containing 34 samples.

Statistical analysis

Analysis of Variance (ANOVA; Statview 3.0) was conducted to determine significant difference and Fisher's protected least squares differences (PLSD) test used to allocate individual differences.

Results

IL-15 expression by cultured ESCs

IL-15 expression increased across the time course from 0 to 72h with both PGE_2 and IFN-γ stimulating an increase in expression (figure 1). Control levels remained low across all time points. When compared to controls the IFN-γ-treated group increased IL-15 mRNA expression at 4, 24 and 72h ($P < 0.05$). The same was the case with the IFN-γ+PGE_2-treated group at all time points ($P < 0.05$). At 72h the PGE_2 treatment increased IL-15 mRNA expression above the control group ($P < 0.005$).

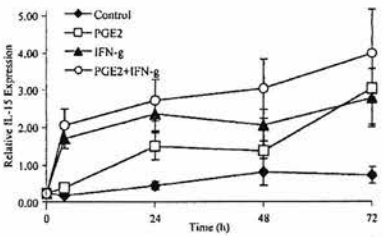

Figure 1. Time course of mRNA IL-15 expression measured by RT-Q-PCR in cultured ESCs (n=4).

IL-15 release from ESCs

Addition of 8-Bromo cAMP created a significant rise ($P < 0.01$) in IL-15 release from ESCs after 3 days and release rose further after 6 days in culture (figure 2). Addition of MPA appeared to augment this response on both days, although not significantly. Cells cultured in

the presence of PGE_2 released higher levels of IL-15 protein into the medium compared to controls on days 3 and 6 but this only became significant when PGE_2 was added in combination with MPA ($P < 0.05$). MPA alone had no effect on IL-15 release compared to controls. IFN-γ alone stimulated a moderate release of IL-15 protein.

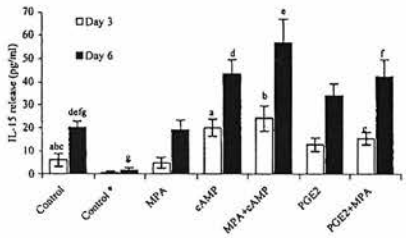

Figure 2. IL-15 protein release from IFN-γ treated human ESCs after 3 and 6 days of treatment. (n=7) * Depicts group with no IFN-γ stimulation. Same letters denote significant difference. [a, d] $P < 0.01$; [b, e] $P < 0.0002$; [c, f, g] $P < 0.05$.

IL-15 expression across the cycle

IL-15 mRNA was detected in all stages of the menstrual cycle and in first trimester decidua but was not present in trophoblast (figure 3). Levels were lowest in the proliferative phase of the cycle. A progressive increase in IL-15 mRNA expression from the proliferative phase to early and mid secretory phases was observed with a dramatic rise in the late secretory phase expressing significantly more than each of the cycle phases ($P < 0.005$). Levels of expression were raised in both menstrual and decidual tissue compared to proliferative tissue although not significantly.

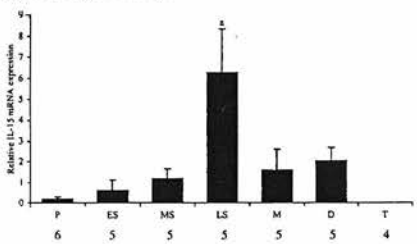

Figure 3. Relative amounts of IL-15 mRNA in proliferative (P), early secretory (ES), mid secretory (MS) and late secretory (LS) endometrium, first trimester decidua (D) and trophoblast (T). [a] $P < 0.005$.

IFN-γ expression across the cycle

Expression of IFN-γ mRNA was detected across the menstrual cycle and in first trimester decidua (Figure 4). Highest levels of expression were found in decidual tissue with a significant elevation in expression ($P < 0.05$) when compared to the early secretory phase.

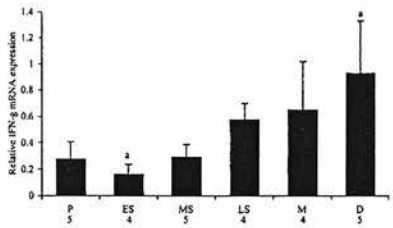

Figure 4. Relative amounts of IFN-γ mRNA in proliferative (P), early secretory (ES), mid secretory (MS) and menstrual (M) endometrium and first trimester decidua (D). [a] $P < 0.05$.

Discussion

The present study demonstrated that PGE_2 and 8-Bromo cAMP were able to increase IL-15 release *in vitro* in the presence of IFN-γ and these responses appeared to be enhanced by addition of a progestin. IFN-γ was essential for this effect and alone could stimulate a small level of IL-15 release from ESCs. However, a progestin alone was not capable of increasing IL-15 protein release from ESCs *in vitro*. Stimulation of ESCs with PGE_2 or IFN-γ separately or in combination, led to an increase in IL-15 mRNA expression *in vitro*. In addition, expression of IL-15 mRNA in first trimester decidua and endometrium across the menstrual cycle was demonstrated. The late secretory stages expressed significantly greater levels ($P < 0.005$) in comparison to all other cycle phases. Expression of IFN-γ mRNA across the menstrual cycle and in decidua was also confirmed. Expression in decidua was significantly greater than that in the early secretory phase ($P < 0.05$).

The expression of IL-15 in a broad spectrum of tissue and cell types, including kidney epithelial cells (13), skeletal muscle (14) and placenta (15) implies that the functions of IL-15 stretch beyond that of the immune system. The possible role of IL-15 in uterine physiology has also recently been explored and the idea of hormonal control over this cytokine proposed (4, 5). The involvement of IL-15 in placental establishment has also been identified (16, 17).

The results of this study suggest that, at the intracellular signalling level (at least *in vitro*), cAMP is a key regulator of IL-15 release from ESCs and that this response is likely to be linked to the action of IFN-γ. The role of IFN-γ in IL-15 release by cells has also been described in the kidney (13, 18). The presence of IFN-γ in human endometrium is a contentious issue although IFN-γ immunoreactivity in lymphoid aggregates and in cells dispersed throughout the stroma has been established (19). In this study we report, quantitative expression of IFN-γ throughout the menstrual cycle and first trimester decidua and demonstrated a rise in expression in first trimester decidua by RT-Q-PCR. Uterine NK cells are a potential

source of IFN-γ and are situated both perivascularly and in close proximity to glands. There is a further infiltration of uterine NK cells throughout the stroma in the late secretory phase (20). This pattern of rising uterine NK cell numbers post-ovulation parallels the IL-15 expression demonstrated across the cycle in this study. It has been proposed that IL-15 is the stimulator of this proliferation (2). These cells are believed to undergo apoptosis prior to menstruation. However, they appear to be "rescued" if pregnancy is established and full decidual transformation completed thus implying a link between uterine NK cells and decidualisation (1).

The present study demonstrated that PGE_2 is able to stimulate IL-15 expression and release by ESCs. PGE_2 can act via the second messenger cAMP and this supports the present observations (9). The responses of both PGE_2 and 8-Bromo cAMP appear to be enhanced by addition of a progestin. This is in agreement with other studies that have suggested progesterone is involved in IL-15 regulation in human endometrium (4, 5). One of the roles of progesterone in this circumstance may be stimulation of the EP2 receptor in ESCs (21). Studies using endometrial explant cultures have compounded evidence for hormonal control of PGE_2. Secretory endometrium released greater levels of prostaglandins than proliferative endometrium and from these results an involvement of PGE_2 in menstruation has been proposed (22, 23). Additionally, synergistic actions between MPA and estradiol plus PGE_2 on the process of *in vitro* decidualisation have previously been reported (10). An up-regulation of IL-15 mRNA expression during *in vitro* decidualisation (6) supports the link between IL-15 and decidualisation. Previous studies have demonstrated a central role for cAMP in increasing prolactin expression, a marker of decidualisation (24, 25).

In conclusion, this study provides evidence of a role for PGE_2 in IL-15 regulation in concert with the actions of IFN-γ and supports the view that IL-15 is closely linked to decidualisation and uterine NK cell function. A greater knowledge about stromal and uterine NK cell interactions in human endometrium will benefit our understanding of key reproductive processes such as decidualisation, menstruation, placental formation and the establishment and maintenance of early pregnancy.

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PROGESTERONE CONTROL OF HUMAN ENDOMETRIAL CELLS

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2004

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Abstract

The coordination of events and cellular interactions within the uterus is vital to the establishment of pregnancy, a process constrained to a narrow window of time within the ovarian cycle. The transformation of the endometrium into decidua is one such event and is considered essential to embryo implantation and to the maintenance of pregnancy. In humans these changes are most dramatic in the stromal compartment and are influenced by progesterone, secreted by the Corpus Luteum (CL). This hormone is believed to be the main factor inducing such differentiation in the oestradiol-primed stroma. However, mediators with the ability to activate the protein kinase A/cAMP pathway, such as Prostaglandin E_2 (PGE_2) and relaxin, are potent inducers of decidualisation when administered alongside progesterone in endometrial stromal cells (ESCs) *in vitro*. Uterine Natural Killer (uNK) cells increase in number in secretory phase endometrial stroma, implying the control of progesterone on their expansion. However, they lack the nuclear progesterone receptor and growth and differentiation may depend on interactions with ESCs. uNK cells replicate upon Interleukin-15 (IL-15) administration *in vitro* and this cytokine is expressed in the epithelial and stromal cells. The aims of this research project have been to investigate *in vitro* decidualisation of ESCs and regulation of IL-15 and uNK cells in order to extrapolate how ESCs and uNK cells may interact during the secretory phase and early pregnancy.

The present study has explored the factors involved in decidualisation using primary human ESC cultures. Quantitative real-time PCR (Q RT-PCR) and Enzyme-linked Immunoabsorbant Assays (ELISA) have been used to investigate effective *in vitro* stimuli of decidualisation. A combination of treatment with a progestin and either 8-Bromo cAMP or PGE_2 was capable of stimulating decidualisation in ESC cultures as determined by increases in two markers of this process, prolactin and insulin-like growth factor-binding protein-1 (IGFBP-1). Further analysis has revealed the changes taking place within the PGE_2 pathway in decidualising ESCs, including an upregulation in the

EP_2 prostaglandin receptor messenger RNA (mRNA) upon treatment with 8-Bromo cAMP plus a progestin.

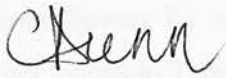
The results present here have demonstrated a rise in IL-15 mRNA levels in parallel with *in vitro* decidualisation. It appears that both progesterone and the intracellular messenger, cAMP, are involved in decidualisation and IL-15 expression. IL-15 secretion from the cells is shown to be IFN-γ dependent. The expression of IL-15 and interferon-γ (IFN-γ) mRNA across the menstrual cycle has been established. Immunohistochemistry was used to determine IL-15 expression during simulated early pregnancy compared with normal luteal controls and has shown that secretions of the CL, including progesterone and/or relaxin, have the ability to increase IL-15 expression *in vivo*. Primary cultures of human uNK and peripheral blood NK cells have been used for studying the T helper 2-type cytokine IL-10 which is believed important for the support of early pregnancy. In response to PGE_2 treatment, uNK cells expressed and secreted raised levels of IL-10, an anti-inflammatory cytokine.

Further investigation into the interactions between the convergence of the cAMP and progesterone intracellular pathways and their receptors would be important in clarifying the exact mechanisms controlling ESC decidualisation and IL-15 regulation. The interactions between ESCs and uNK cells need to be clarified further to assess the roles of uNK cells in reproductive processes.

Contents

Declaration

Except where due acknowledgement is made by reference the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.



Carolyn Dunn

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Many thanks to the friends I have made at the Human Reproductive Sciences Unit and finally a special thank you to my family, who even from a distance, have been invaluable support and encouragement.

Abbreviations

AA	arachidonic acid
ABC	avidin biotin peroxidase detection system
ANOVA	analysis of variance
ART	assisted reproductive technology
AS	anti-sera
ATP	adenosine triphosphate
α-2 M	α-2 macroglobulin
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
cAMP	adenosine-3',5'-cyclic monophosphate
CAT assay	chloramphenicol acetyl transferase
CD	cluster determinant
CD40L	CD40 ligand
cDNA	complimentary DNA
c-fms	receptor of M-CSF
CL	corpus luteum
COX-1/2	cyclo-oxygenase-1/2
CSF-1	colony stimulating factor-1
CSM	cell separation medium
Ct	threshold cycle
DAB	3,3'-diaminobenzidine
DEPC	Diethyl pyrocarbonate
dH_2O	distilled water
DSC	decidualised stromal cell
ECM	extracellular matrix
EDTA	ethylenediaminetetracetic acid
EGF	epidermal growth factor
ELISA	enzyme linked immunoabsorbant assay

EP_{1-4}	PGE_2 receptors 1-4
ER-α/β	oestrogen receptor-α/β
ESC	endometrial stromal cell
FACS	fluorescent activated cell sorter
FAM	6-carboxy-fluorescein
FCS	fetal calf serum
FSH	follicle stimulating hormone
FITC	fluorescein isothiocyanate conjugate
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte/macrophage-colony stimulating factor
GPCR	G-protein coupled receptor
HBD 1-4	human beta defensins 1-4
hCG	human chorionic gonadotrophin
HLA-DR	human leukocyte antigen
ICAM	inflammatory cell adhesion molecule
IFN-α/β/γ/τ	interferon-α/β/γ/τ
IGF	insulin-like growth factor
IGFBP-1/2 etc	insulin-like growth factor binding protein-1/2 etc
IgG	immunoglobulin
IL-1/2 etc	interleukin-1/2 etc
IL-1R/11R etc	IL-1/11 receptor etc
iNOS	inducible nitric oxide synthase
IRF-1	interferon regulatory factor-1
IUGR	intra uterine growth restriction
KDR	Type 2 receptor of VEGF
LGL	large granular lymphocyte
LH	luteinising hormone
LIF	leukaemia inhibitory factor
LMP	last menstrual period
LNG-IUS	levonorgestrel-releasing intra uterine system

LPD	luteal phase defect
LSP	long signal peptide
MCP-1	monocyte chemoattractant protein
M-CSF	macrophage-colony stimulating factor
MHC	major histocompatibility complex
MIP-1α	macrophage inflammatory protein-1α
MoxB	methyl oximating B solution
MP	multi-purpose
MPA	medroxyprogesterone acetate
MMP	matrix metalloproteinase
MRP-4	multi-drug resistant protein-4
NBF	neutral buffered formalin
NFκB	nuclear factor κ B
NHS	normal horse serum
NK	natural killer (cell)
NSAIDs	non-steroidal anti-inflammatory drugs
NSB	non-specific binding
PB	peripheral blood
PBMC	peripheral blood monocyte cells
PBS	phosphate buffered saline
PDE3a/4b	phophodiesterase3a/4b
PG	prostaglandin
PGD_2	prostaglandin D_2
PGDH	prostaglandin dehydrogenase
PGH_2	prostaglandin H_2
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostaglandin I_2
PGTP	prostaglandin transport protein
PKA	protein kinase A

PLA_2	phospholipase A_2
PLSD	protected least squares difference
PR-A/B/C	progesterone receptor-A/B/C
PMA	phorbol 12-myristate 13-acetate
Prl	prolactin
Prl-R	prolactin receptor
Q RT-PCR	quantitative reverse transcription-polymerase chain reaction
RCLB	red blood cell lysis buffer
RIA	radioimmunoassay
rpm	revolutions per minute
RSB	RNA storage buffer
RU486	mifepristone
SLPI	secretory leukocyte protease inhibitor
SPC	streptavidin peroxidase conjugate
SSP	short signal peptide
TBS	tris buffered saline
TGF-β	transforming growth factor-β
Th-1/2	T helper-1/2
TIMP	tissue inhibitor of MMP
TNF-α	tumour necrosis factor-α
TXA_2	thromboxane A_2
uNK	uterine natural killer (cell)
VEGF	vascular endothelial growth factor

1. Literature Review

1.1 The Uterus and the Menstrual Cycle

The uterus has the role of supporting and retaining the fetus throughout pregnancy. The organ is lined by a glandular tissue layer termed the endometrium and in the non-pregnant state the functional layer of the human endometrium undergoes cyclical regeneration and degeneration. This takes place over a 28 day period and is primarily determined by the ovarian hormones, oestrogen and progesterone. A progressive and sequential maturation of the endometrium is essential preparation for implantation of the blastocyst. There are three major phases, namely menstruation, proliferation and secretion (figure 1.1). The secretory phase is surprisingly highly regular in length, even if there is individual variation in proliferative phase length. The functional layer of the endometrium is shed via the process of menstruation if implantation fails to occur. This layer is then replenished from the basal endometrial layer and the process begins once more. The major cyclical changes in response to progesterone and oestrogen have been described by Noyes *et al* 1950 (Noyes *et al.*, 1950) (figure 1.2).

1.1.1 The Proliferative Phase

The proliferative phase lasts from day 4 to day 14 of the cycle and is oestrogen dominated. Oestrogen receptors are present in the glandular and stromal compartments as are progesterone receptors during the follicular phase (Lessey *et al.*, 1988; Snijders *et al.*, 1992; Wang *et al.*, 1998). The primary source of oestrogen is the developing Graaffian follicle. Follicle stimulating hormone (FSH) released from the pituitary gland stimulates maturation of one or more of these follicles. The oestrogen released stimulates proliferation of the endometrium and this is reflected by a variety of changes. New glands form and are straight and tubular in structure and along with the stroma, regenerate from the residual basal endometrium. The nuclei of the glands become prominent and increase in mitotic activity. In the compact stromal compartment mitoses are also abundant. The stroma becomes highly vascularised and the arteries become

spiral in formation. As the luteinising hormone (LH) surge on day 14 approaches the glands become pseudo-stratified and tall and columnar in shape.

1.1.2 The Secretory Phase

The secretory phase commences from day 16 to 25 and is progesterone dominated. The continued release of LH from the pituitary induces the luteinisation of the theca and granulosa cells of the corpus luteum (CL). The granulosa lutein cells secrete progesterone, maintaining the secretory transformation of the endometrium.

In the early secretory phase the glands develop subnuclear vacuoles of glycogen and they become convoluted. The gland mitoses decrease at this point and are absent after day 18. The glandular glycogen deposits begin to appear on the apical side of the cells around day 18 of the cycle and continues to increase with maximal secretions being released around days 20 and 21 before exhausting their stores. The tortuosity of the glands increases in the mid to late secretory phase. Stromal oedema increases across the secretory phase, peaking in the mid-section. During the mid to late secretory phase the stromal cells begin to change both structurally and functionally. This predecidual change is initially observed around the spiral arterioles before it spreads to other regions of the stroma. The stromal cells undergo a decidual reaction in preparation for implantation and progressively forms the decidua during pregnancy. Pinopodes are expressed on days 19-21 on the apical surface of the luminal uterine epithelium. The timing of their appearance is progesterone-dependant and they appear around the time of implantation (Martel *et al.*, 1991; Nikas *et al.*, 1995; Nikas *et al.*, 1997).

In the late secretory phase, an infiltration of leukocytes invades the endometrium. Although macrophages and lymphocytes accumulate, the major leukocyte cell type in the secretory phase is the uterine Natural Killer (uNK) cell. These cells remain abundant in the first trimester of pregnancy and will be discussed in more detail in section 1.2.2.

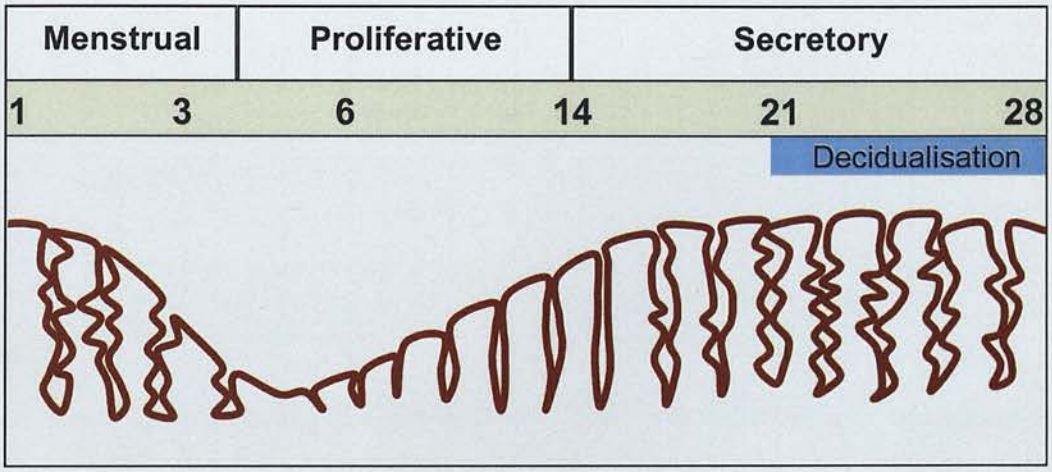
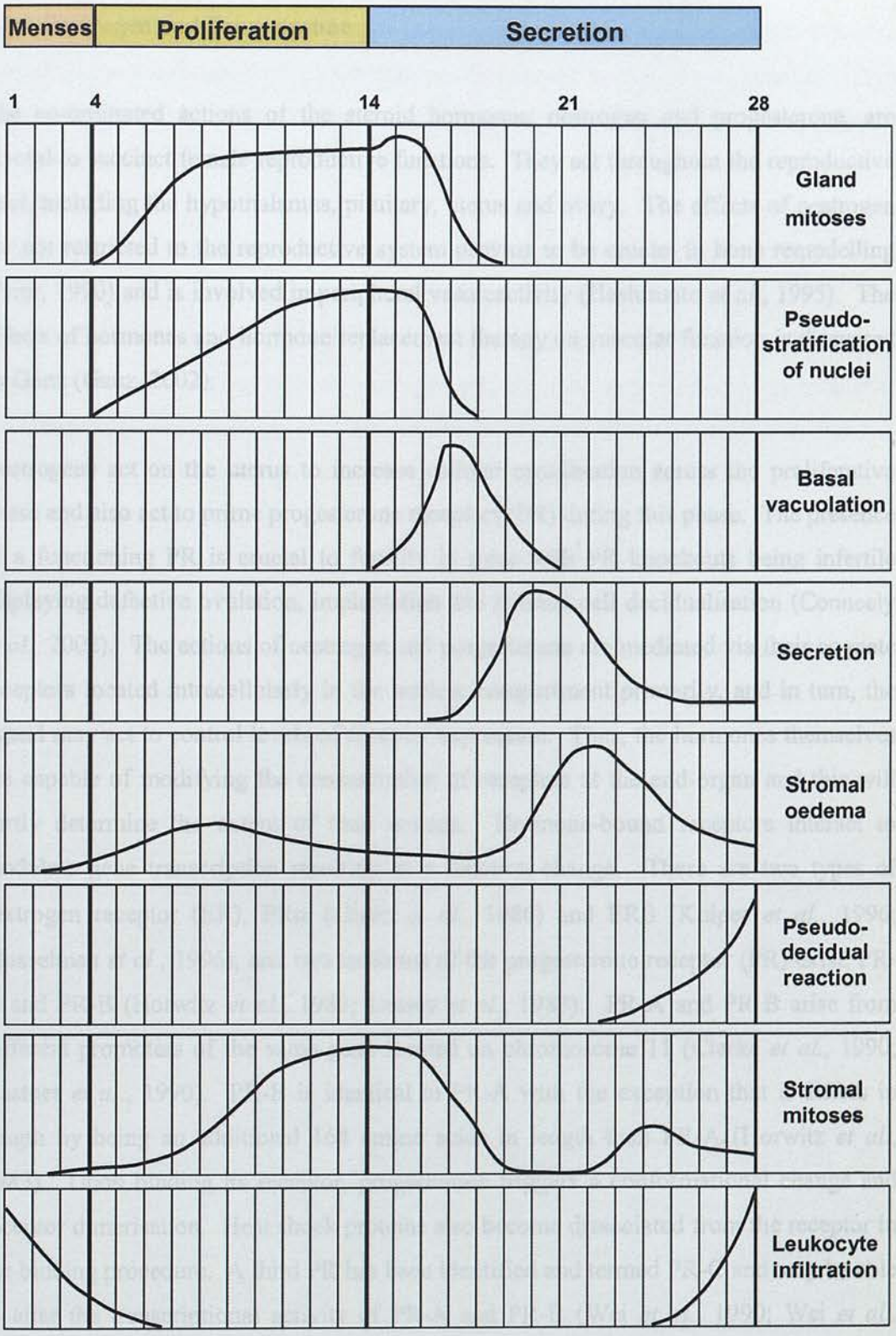


Figure 1.1

Diagrammatic representation of the cyclical nature of degeneration and regeneration of the functional endometrial layer with the major stages being highlighted. This is based on the "classic" 28 day cycle in humans.

Figure 1.2

The changes that take place in the human endometrium across the menstrual cycle based on the "classic" 28 day model as described by Noyes *et al* 1950 (Noyes *et al.*, 1950).


Menses
Proliferation
Secretion
1
4
14
21
28
Gland mitoses
Pseudo-stratification of nuclei
Basal vacuolation
Secretion
Stromal oedema
Pseudo-decidual reaction
Stromal mitoses
Leukocyte infiltration

1.1.3 Oestrogen and Progesterone

The co-ordinated actions of the steroid hormones, oestrogen and progesterone, are pivotal to succinct female reproductive functions. They act throughout the reproductive tract, including the hypothalamus, pituitary, uterus and ovary. The effects of oestrogen are not restricted to the reproductive system proving to be crucial in bone remodelling (Prior, 1990) and is involved in peripheral vasoreactivity (Hashimoto *et al.*, 1995). The effects of hormones and hormone replacement therapy on vascular function is discussed by Ganz (Ganz, 2002).

Oestrogens act on the uterus to increase cellular proliferation across the proliferative phase and also act to prime progesterone receptors (PR) during this phase. The presence of a functioning PR is crucial to fertility in mice with PR knockouts being infertile displaying defective ovulation, implantation and stromal cell decidualisation (Conneely *et al.*, 2002). The actions of oestrogen and progesterone are mediated via their cognate receptors located intracellularly in the nuclear compartment primarily, and in turn, the ligand may act to control levels of receptor expression. Thus, the hormones themselves are capable of modifying the concentration of receptors at the end-organ and this will partly determine the extent of their actions. Hormone-bound receptors interact to modulate gene transcription resulting in a function change. There are two types of oestrogen receptor (ER), ERα (Green *et al.*, 1986) and ERβ (Kuiper *et al.*, 1996; Mosselman *et al.*, 1996), and two isoforms of the progesterone receptor (PR) exist, PR-A and PR-B (Horwitz *et al.*, 1983; Lessey *et al.*, 1983). PR-A and PR-B arise from different promoters of the same gene located on chromosome 11 (Clarke *et al.*, 1990; Kastner *et al.*, 1990). PR-B is identical to PR-A with the exception that it differs in length by being an additional 164 amino acids in length than PR-A (Horwitz *et al.*, 1983). Upon binding its receptor, progesterone triggers a conformational change and receptor dimerisation. Heat shock proteins also become dissociated from the receptor in the binding procedure. A third PR has been identified and termed PR-C and may be able to alter the transcriptional activity of PR-A and PR-B (Wei *et al.*, 1990; Wei *et al.*,

1996). In addition, more recently a fourth novel, truncated PR has been cloned from human adipose and aortic cDNA libraries and is believed to have a non-genomic action (Saner *et al.*, 2003). The functional importance of this receptor with context to the endometrium is currently under investigation.

1.1.3.1 Receptor Cross-talk

Communication also takes place between the oestrogen and progesterone receptors. PRs are under the dual control of both oestrogen and progesterone. Experiments involving rat uterine cells demonstrated that when either a progestin or anti-progestin is bound to either PR-A or -B a resulting inhibition in oestradiol's ability to stimulate ER activity is reported (Katzenellenbogen, 2000). Exposure of the endometrium to high dose progestins, as with the Levonorgestrel-releasing intrauterine system (LNG-IUS), down-regulates the ER and PR-A and PR-B in both the glands and stroma (Critchley *et al.*, 1998b) implying a dependency of ER levels on progesterone and its receptors.

1.1.3.2 Immunohistochemical Localisation

Immunohistological studies within the human endometrium have described the cyclical variation of oestrogen (ER) and progesterone receptor (PR) levels in both functional and basal regions and within the epithelial and stromal compartments (Garcia *et al.*, 1988; Lessey *et al.*, 1988; Critchley *et al.*, 1993; Snijders *et al.*, 1996; Wang *et al.*, 1998) (Summarised in table 1.1). They have demonstrated the nuclear locality of both steroid receptors. PR-A and PR-B are located in the glands and stroma during the proliferative phase but only the PR-A isoform is present in the functional layer across the secretory phase and early pregnancy and is localised to the stromal cells (Wang *et al.*, 1998). This implies that PR-A is responsible for the luteal phase actions of progesterone within the stroma and in particular on decidualisation. Immunoexpression of ERα increases in the functional layer across the proliferative phase and reaches a peak in the late proliferative

endometrium. The ER in the glands is down-regulated in the secretory phase by the rising progesterone levels. This reduction of ER can be blocked by administration of the anti-progestin, RU486, when applied early in the secretory phase confirming the role of progesterone (Maentausta *et al.*, 1993; Cameron *et al.*, 1996). A decline in ERα occurs in glandular and stromal compartments of the functional layer across the secretory phase. *In situ* hybridisation has located the two ER isoforms within the human endometrium (Matsuzaki *et al.*, 1999). The same expression pattern for ERα was observed as with the immunohistological studies. ERβ mRNA was detected in both glands and stroma with decreased expression in the glandular region in the late secretory phase functional layer (Critchley *et al.*, 2001a; Lecce *et al.*, 2001). The ERβ was also localised to endothelial cells implying an influence on vascular regulation. In women with compromised fertility and receiving clomiphene citrate "anti-oestrogen" therapy, the low pregnancy rates achieved were correlated with low oestrogen receptor concentrations in the preovulatory endometrium (Ohno *et al.*, 1998).

A study *in vitro* has shown that during decidualisation the PR-A is more abundant than PR-B. However, they found a reduction in the PR-A with progression of the decidualisation process and this reduction was accelerated by addition of a synthetic progestin (Brosens *et al.*, 1999). In addition, transient transfection of either PR-A or PR-B caused an inhibition of decidual prolactin (Prl) promoter-reporter construct in response to cAMP. Following insertion of LNG-IUS both isoforms of the PR are downregulated in the glands and the stroma (Critchley *et al.*, 1998b) implying regulation of both isoforms by their ligand. It appears to be PR-A that is essential to uterine and ovarian reproductive processes, at least in the murine uterus (Conneely *et al.*, 2002). They discovered that in the chick oviduct PR-A and PR-B are produced by translation at two distinct AUG signals encoded by a single gene and could therefore selectively ablate either of these isoforms (Conneely *et al.*, 1987). Mouse knock-outs for PR-A exhibited infertility, whilst the necessity of the PR-B isoform is restricted to the mammary gland (Conneely *et al.*, 2002).

	Proliferative phase		Secretory phase	
	Glands	**Stroma**	**Glands**	**Stroma**
Functional				
PR-A+B	++	++	-	++
PR-B	++	++	-	-
ERα	++	++	-	-
ERβ	++	++	++(+)	++
Basal				
PR-A+B	++	++	+	++
ERα	++	++	+	+
ERβ	++	++	++	++

Table 1.1

Relative immunostaining intensity for oestrogen and progesterone receptors in the functional and basal endometrium across the proliferative and secretory phases of the cycle. Negative immunostaining is represented by (-), positive immunostaining is represented by (+) or (++) depending on intensity. Adapted from Critchley 2000 (Critchley, 2000).

	Proliferative phase		Secretory phase	
	Glands	**Stroma**	**Glands**	**Stroma**
Functional				
PR-A+B	++	++	-	++
PR-B	++	++	-	-
ERα	++	++	-	-
ERβ	++	++	++(+)	++
Basal				
PR-A+B	++	++	+	++
ERα	++	++	+	+
ERβ	++	++	++	++

1.1.4 Regulation of Menstruation

If implantation fails to occur, the functional layer of the endometrium is shed by the process of menstruation in response to declining progesterone levels as a result of CL demise. The work of Markee 1940 on the rhesus monkey provides much of our present understanding of the vascular changes occurring during menstruation with the determination that progesterone and oestrogen are major controlling factors over these structural changes. It was known that progesterone acts following oestrogen priming and then is withdrawn in the lead up to menstruation. The effects of these hormonal changes were studied in endometrial fragments transplanted into the eye of the rhesus monkey, where changes in the vasculature could be observed (Markee, 1940). Vessels become coiled across the luteal phase to form the spiral arterioles and upon withdrawal of progesterone these become vasodilated followed by vasoconstriction (Markee, 1940). This results in lesions and breakdown of the tissue. The vasoactive products found in menstrual fluid have been identified as being PGE_2 and $PGF_{2\alpha}$ (Lumsden *et al.*, 1983). Hormones have been implicated in regulating production of these prostaglandins (PG) in the human endometrium via explant studies (Abel *et al.*, 1980; Abel *et al.*, 1983) and a more detailed account of their regulation is discussed in section 1.4.1. $PGF_{2\alpha}$ is a vasoconstrictive agent and has been implicated in the initiation of menstruation (Baird *et al.*, 1996). During the mid luteal phase prostaglandin dehydrogenase activity (PGDH) is high relative to the proliferative phase and therefore mediates metabolism of PGE_2 and $PGF_{2\alpha}$ (Casey *et al.*, 1980; Critchley *et al.*, 1998a).

1.1.4.1 Menstruation and Inflammation

The process of menstruation appears to be multi-factorial and in addition to hormonal control, the process is considered to be an inflammatory event (Finn, 1986; Kelly *et al.*, 1994). Leukocytes infiltrate into the endometrium in the pre-menstrual phase, in addition to providing defence against pathogens at this vulnerable time, they are also likely to be involved directly in tissue breakdown via production of proteases, and

indirectly by the release of chemokines and cytokines. Infiltration and the roles of these different cells will be discussed in section 1.2.1. These leukocytes include macrophages, neutrophils and uNK cells and all lack genomic steroid receptors (Poropatich *et al.*, 1987; Henderson *et al.*, 2003) and therefore paracrine control over these cells must be occurring. Neutrophils are expressed across the cycle in very low numbers but rise dramatically in the immediate premenstrual phase to sites of uterine inflammation (Noyes *et al.*, 1950; Kamat *et al.*, 1987; Poropatich *et al.*, 1987). An important chemotaxis stimulus for neutrophils is IL-8 (neutrophil chemotactic factor) (Rampart *et al.*, 1989; Colditz *et al.*, 1990).

1.1.4.2 Matrix Metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are enzymes released by both the invading immune cells and by the stromal cells. The breakdown of the extracellular matrix has been attributed to the actions of MMPs (Schatz *et al.*, 1997; Lockwood *et al.*, 1998; Salamonsen *et al.*, 1999) and their role in menstruation implied by the timing of their expression within the human endometrium (Rodgers *et al.*, 1993; Hampton *et al.*, 1994; Rodgers *et al.*, 1994). MMPs are likely to be under progesterone-control since withdrawal of progesterone in cultures of endometrial stromal cells (ESCs) enhances proMMP-2 (Irwin *et al.*, 1996) and proMMP-3 (stromelysin-1) production (Schatz *et al.*, 1994). A culture system of human ESCs was used to mimic the luteal phase and demonstrated upregulation of MMP-1, -2, -3 and -4 in latent form upon progesterone withdrawal (Salamonsen *et al.*, 1997). Their tissue inhibitors (TIMPs) were also analysed and showed no regulation of TIMP-1, -2 and -3 upon withdrawal of progesterone, thus implicating a greater complexity to MMP regulation beyond that of being solely progesterone-determined. Leukocytes are another source of MMPs and via stromal and epithelial interactions, MMP release and action from this source may be being controlled (Salamonsen *et al.*, 2000).

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1.1.4.3 Vascular Endothelial Growth Factor (VEGF) and Hypoxia

The type 2 receptor for vascular endothelial growth factor (VEGF), KDR, is expressed only in endometrial endothelial cells until the premenstrual phase when expression is observed in the superficial stroma (Nayak *et al.*, 2000). VEGF is expressed across the menstrual cycle (Zhang *et al.*, 1998a) and is upregulated during the menstrual phase via a hypoxic stimulus (Sharkey *et al.*, 2000). Hypoxia has been shown to raise VEGF levels in cultures of human ESCs (Popovici *et al.*, 1999) and the presence of the response element for the hypoxia inducible factor has been located on the VEGF promoter (Goldberg *et al.*, 1994). *In vitro* decidualised ESCs express greater levels of VEGF compared with controls using Microarray Technology (Popovici *et al.*, 2000). VEGF has been shown to increase the expression of MMPs (Unemori *et al.*, 1992; Wang *et al.*, 1998) and may be acting as an additional control on MMP production during the premenstrual phase (Nayak *et al.*, 2000; Critchley *et al.*, 2001b).

As discussed by Salamonsen *et al* 1999 menstrual degeneration is focal and not ubiquitous throughout the endometrium, with the regeneration and repair of the endometrium beginning to occur 36 hours after the onset of menstruation. During this repair process no scarring is evident as with normal tissue repair although the exact mechanisms are not yet understood it may be relevant in understanding pathologies such as menorrhagia (Salamonsen *et al.*, 1999).

1.1.5 Decidua and Pregnancy

Decidualisation is unique to species that undergo haemochorial placentation and in the human endometrium is considered an essential pre-requisite for establishment of pregnancy. The decidua is the transformed endometrium that is morphologically and functionally distinct. The endometrial cells differentiate and an infiltration of lymphoid cells occurs. Specific products are secreted by decidual cells, for example prolactin and IGFBP-1, and their potential functions, with regard to pregnancy, are discussed in

section 1.3.1.1. With regard to humans it is difficult to assess the direct necessity of decidualisation to implantation. However, it occurs in every menstrual cycle in advance of implantation and it may be that products of decidua are involved in this process. Conditions involving disturbance of stromal differentiation, such as endometriosis and luteal phase defect (LPD) can result in reduced fertility and menstrual irregularity (Vanrell *et al.*, 1986; Ronnberg, 1990; Lessey, 2002) and this evidence implicates decidualisation in being critical to the implantation process. LPD affects 3-4% of infertile women and can be defined as a lag of greater than 2 days histological development compared to the cycle day. This condition is believed to be due to either inadequate action or secretion progesterone, an essential stimulus for decidualisation. Decidual cells may play significant roles in controlling the extent of trophoblast invasion (Pijnenborg *et al.*, 1981). If implantation occurs in regions deficient of decidua, due to scar tissue, over-invasion occurs and conversely if under-invasion occurs the result could be the development of pre-eclampsia. Recently alpha-2 macroglobulin (α-2M), a product of endometrial endothelial cells, has been shown to be involved in decidual regulation of trophoblast invasion in mice (Esadeg *et al.*, 2003).

A 50% pregnancy loss occurs at the time of implantation and therefore it is apparent that this is a critical time for the endometrium to either continue with decidualisation or to breakdown by the process of menstruation. Decidual cells are also thought to be important in preventing uterine bleeding in the peri-implantation phase of the cycle and have been associated with spontaneous abortion and pre-term birth (Schatz *et al.*, 2001). Decidualisation initially occurs in the perivascular stromal cells and it may be that these decidual cells are involved in the control of menstruation (Kelly *et al.*, 2002). This close association with the blood vessels may allow control over the vasculature and the decision to stabilise or degenerate. These cells have been shown to produce IL-8 (Critchley *et al.*, 1994) thus stimulating neutophil influx and tissue breakdown via production of enzymes. The decision of the endometrium to breakdown or to fully differentiate is likely to be related to secretion of cytokines and other factors during implantation.

1.2 The Uterine Immune Environment

1.2.1 Leukocyte Infiltration in the Endometrium

According to the criteria of Noyes *et al* 1950, there is an infiltration of leukocytes in the late secretory phase (Noyes *et al.*, 1950) (figure 1.2). The major leukocyte populations located in human endometrium and decidua are macrophages, T cells, B cells and uNK cells and the uNK cell numbers alter in a cyclical manner (Loke *et al.*, 1997). The proliferative phase and early secretory phases have a relatively low abundance of leukocytes, as demonstrated by immunohistochemical staining with the surface marker, CD56, with an increase in the late secretory phase and into early pregnancy (Bulmer *et al.*, 1991). In addition to these CD56 positive leukocytes, an influx of neutrophils occurs in the perimenstrual period at which point they comprise approximately 6-15% of total cells, and they are thought to be important in menstruation (Salamonsen *et al.*, 1999). Eosinophils also rise immediately prior to menstruation and make up around 3-5% of endometrial cells at this point (Salamonsen *et al.*, 2000). Macrophages show a small rise in number from the proliferative phase to the secretory phase (Bonatz *et al.*, 1992) although their expression of MHC class II does not appear to vary (Bulmer *et al.*, 1988). Macrophages are also raised in number in endometrium taken from women 48 hours after controlled progesterone withdrawal compared to decidua from pregnant women (Critchley *et al.*, 1999). T and B lymphocytes are located in the basal layer in lymphoid aggregates and do not vary in number across the menstrual cycle (Bulmer *et al.*, 1991). In addition, T lymphocytes are also located throughout the functional layer in intra-epithelial locations (Loke *et al.*, 1995). These cells become activated during pregnancy at the site of large lymphoid cell clusters and it is possible they are acting to provide constitutive immune defence at this critical time or control trophoblast invasion (Mincheva-Nilsson *et al.*, 1994).

How these immune cells are regulated with regard to their recruitment in the human endometrium has not been fully established. Cells may be migrating from peripheral blood (Marzusch *et al.*, 1993) or be proliferating within the tissue itself (Klentzeris *et al.*, 1992). Endometrial chemokines may be responsible for the up-regulation of monocytes in the secretory phase (Kamat *et al.*, 1987) and high numbers of these cells are observed around the time of implantation (Bonatz *et al.*, 1992). Chemokines are chemotactic cytokines that act via G protein-coupled receptors (GPCRs) (Murphy, 1996) and their roles as chemoattractants for leukocytes will be discussed in this section. Chemokines consist of four cysteine residues and two disulphide bonds and are split into four sub-groups according to the separation of the first two cysteines by amino acids: C, CC, CXC and CXXXC (Zlotnik *et al.*, 2000).

1.2.1.2 Interleukin-8 (IL-8)

In addition to chemotaxis, IL-8 is implicated in angiogenesis (Koch *et al.*, 1992) and mitogensis of epidermal cells (Tuschil *et al.*, 1992). In the human uterus, IL-8 has been located to the perivascular cells in the late secretory phase and first trimester decidua (Critchley *et al.*, 1994; Critchley *et al.*, 1996; Critchley *et al.*, 1999; Milne *et al.*, 1999). It is also present in choriodecidual cells (Kelly *et al.*, 1992) and in amniotic fluid (Laham *et al.*, 1993). In endometriotic tissue it fails to vary with the menstrual cycle implicating persistent recruitment of leukocytes in this pathological tissue that may be under IL-8 control (Akoum *et al.*, 2001). Cultures of human ESCs and epithelial cells have demonstrated that IL-8 production can be regulated by IL-1α and TNFα (Arici *et al.*, 1993). Cell surface peptidases such as aminopeptidase N (APN) are expressed in human endometrium and their regulation of growth factors, peptide hormones and growth factors has been suggested previously (Imai *et al.*, 1992; Imai *et al.*, 1996). It has been proposed that IL-8 levels *in vivo* may be controlled via APN (Seli *et al.*, 2001). Progesterone is thought to be a main regulator of IL-8 expression. In human endometrial explant studies, progesterone acted to inhibit IL-8 secretion (Kelly *et al.*, 1994). This is in agreement with *in vivo* data that demonstrates an increase in IL-8

immunostaining in human endometrium 48 hours after progesterone withdrawal in a study designed to mimic the late secretory phase (Critchley *et al.*, 1999). This implicates IL-8 for a role in menstruation but it has also been proposed as a cervical ripening agent (Kelly *et al.*, 1992). IL-8 is able to synergise with PGE_2 to recruit neutrophils (Rampart *et al.*, 1989; Colditz *et al.*, 1990) which in turn aid the breakdown of collagen therefore softening the cervix at labour (Junqueira *et al.*, 1980).

1.2.1.3 Monocyte Chemotatic Protein-1 (MCP-1)

Monocyte chemotactic protein-1 (MCP-1) is an example of a CC chemokine and is a product of an array of cell types including fibroblasts (Yoshimura *et al.*, 1990), endothelial cells (Sica *et al.*, 1990) and monocytes (Yoshimura *et al.*, 1989) and, as its name implies, is chemotactic for monocytes (Rollins *et al.*, 1991). MCP-1 is co-expressed along with IL-8 in perivascular cells in the human endometrium in the late secretory phase (Jones *et al.*, 1997). This study also confirmed that the expression of these two chemokines coincides with COX-2 expression and implicates these factors in perimenstrual functions. Cultures of human ESCs and epithelial cells secrete MCP-1 (Arici *et al.*, 1995) and have been shown to respond to the T helper-1 cytokine, IFN-γ, by releasing MCP-1 after 24 hours of treatment (King *et al.*, 2001). Oestrogen has been shown to inhibit its secretion by ESCs (Arici *et al.*, 1999). MCP-1 production by choriodecidual cells and the breast cancer cell line, T47D, is suppressed by progesterone (Kelly *et al.*, 1997) further implicating a hormonal control over MCP-1. However, a more recent study failed to show the inhibitory effects of progesterone on either MCP-1, -2 or –3 mRNA levels in cultured human ESCs or epithelial cells (DeLoia *et al.*, 2000).

1.2.2 Uterine Natural Killer (uNK) Cells

Natural Killer cells are lymphocytes and have been shown to be critical in innate immune host defence (Bancroft, 1993; Scharton-Kersten *et al.*, 1997; Biron *et al.*, 1999; Cooper *et al.*, 2001b). They are present in peripheral blood, accounting for

approximately 10% of total blood lymphocytes (Robertson *et al.*, 1990). There is one important division with category according to their cell surface marker expression as either $CD56^{bright}$ $CD16^{-}$ or $CD56^{Dim}$ $CD16^{+}$. However, the $CD56^{bright}$ variety is scarce, comprising of less than 2% of total blood lymphocytes and approximately 10% of total blood NK cells (Lanier *et al.*, 1986). Upon activation $CD56^{bright}$ NK cells produce an array of cytokines such as IFN-γ and GM-CSF, (Cooper *et al.*, 2001a) and also develop cytotoxic activity upon IL-12 or IL-2 activation (Ellis *et al.*, 1989; Gately *et al.*, 1991; Robertson *et al.*, 1992). It has been demonstrated that the $CD56^{bright}$ NK variety produce far greater levels of cytokines compared with $CD56^{Dim}$ cells (Cooper *et al.*, 2001a).

1.2.2.1 Uterine Expression and Functions of uNK Cells

Within the uterus, NK cells were originally termed Large Granular Leukocytes (LGLs) due to their size and abundance of cytoplasmic granules (King *et al.*, 1991). They express CD56 on their cell surface making them distinct from the major blood NK cell population. However, a study comparing cytokine production by peripheral NK cells and decidual NK cells reported a very similar cytokine repertoire (Saito *et al.*, 1993). Through their secretion of cytokines it is likely they are having immunoregulatory effects. Their density is low throughout the proliferative phase but their abundance increases across the secretory phase, particularly around the time of implantation (Loke *et al.*, 1997). In the late secretory phase they comprise approximately 15–25% of the ESCs (King *et al.*, 1989b). Peripheral NK cell number is raised in women with recurrent spontaneous miscarriage of unknown aetiology, and this implies a role in controlling pregnancy maintenance (Ntrivalas *et al.*, 2001). uNK cell levels remain high into the first trimester of gestation but then decline and they also disappear in the non-pregnant endometrium prior to menstruation, possibly undergoing apoptosis (King, 2000). The first trimester appears to be a critical time for a successful pregnancy with highest miscarriage rates prevailing in this stage with approximately 50% of total human conceptions failing before the first missed menstrual period. The exact functions of these uNK cells in humans have yet to be elucidated.

How uNK cells are regulated is not yet fully understood. Their expansion across the secretory phase when progesterone levels are rising implicates progesterone as a central regulator of their growth and this is compounded by evidence that ovariectomised women lack uNK cells (Loke *et al.*, 1995). However, uNK cells lack the genomic PR and the ERα (King *et al.*, 1996), although they do express ERβ and the glucocorticoid receptor (Henderson *et al.*, 2003). This absence of the genomic PR suggests they are not controlled directly by progesterone across the secretory phase but that it is likely that regulation is via paracrine signalling with non-leukocyte ESCs since these cells persist to express the PR-A isoform at this stage of the cycle (Wang *et al.*, 1998). Studies *in vitro* have demonstrated that uNK cells proliferate upon treatment with IL-15 but other, as of yet, unidentified products of ESCs appear to increase their proliferation further (Verma *et al.*, 2000). Murine uNK cells express mRNA for an array of cytokines including CSF-1, TNF-α, IL-1, LIF and TGF-β (Croy *et al.*, 1991). A study on isolated human decidual NK cells revealed these cells express mRNA and secrete the protein for G-CSF, GM-CSF, M-CSF and LIF (Saito *et al.*, 1993). This demonstrates that a role, in addition to or in place of an immune function, exists for uNK cells although the function of these cytokines remains unclear. A close association exists between uNK cells and trophoblast cells *in vivo* providing circumstantial evidence for a role in trophoblast invasion (Loke *et al.*, 1997). In culture experiments, isolated human cytotrophoblast cells have been shown to attract $CD56^{bright}$ cells via secretion of Monocyte Inflammatory Protein 1α (MIP-1α) and implies a role for MIP-1α in attracting uNK cells (Drake *et al.*, 2001). This is in contrast to the situation in mice where those genetically-ablated for MIP-1α show no difference to wild type mice with regard to uNK cell density at the implantation site (Chantakru *et al.*, 2001). However, in mice, uNK cells are present only in the metrial gland (Croy *et al.*, 1993), the triangle at the apex of the placenta where blood vessels feeding the placenta are close together. In contrast, in humans uNK cells are present throughout the endometrium and it may therefore be necessary to "attract" these cells specifically towards the implantation site.

1.2.2.2 uNK Cells and Decidualisation

uNK cells may be linked with decidualisation. These cells were found to be specifically located in regions of stroma exhibiting pseudodecidual alterations (Bulmer *et al.*, 1988). It has been proposed that uNK cells are required in mice for maintenance of the decidual reaction rather than its initiation (Croy *et al.*, 2002). Evidence from studies on mice lacking IFN-γ implicates this cytokine, a major product of uNK cells, in the decidual reaction and conversion of the uterine vasculature (Ashkar *et al.*, 1999; Ashkar *et al.*, 2000). Rat splenocytes can be primed by prolactin to express the IL-2 receptor and therefore allows these cells to respond to IL-2 stimulation (Mukherjee *et al.*, 1990). In the human endometrium prolactin is secreted by decidualised stromal cells and mRNA expression of its receptor on these cells is under progesterone control (Tseng *et al.*, 1999). A recent study has illustrated uNK cells as a novel target for prolactin action by detection of the prolactin receptor and this may represent a functional link between uNK cells and ESCs, which produce prolactin upon decidualisation, and provide the indirect connection between progesterone control on uNK cells (Gubbay *et al.*, 2002). PGE_2 has inhibitory actions on NK cell IL-15 receptors via down-regulation of the common γ-chain (Joshi *et al.*, 2001). PGE_2 has also been shown to reduce IL-2 receptor expression on NK cells and reduce their proliferation and activity (Parhar *et al.*, 1989). Using IL-2 activated mouse uNK cells, PGE_2 stimulated an increase in the size and granularity of the cells and enhanced the proportion of 4H12 expressing cells (Linnemeyer *et al.*, 1993). The uNK cells accumulating at implantation sites immuno-stain for the 4H12 antibody and they were able to demonstrate in culture that these cells were less cytotoxic than NK cells negative for 4H12. The described effects of PGE_2 are likely to be mediated via cAMP since this second messenger was able to augment the same responses.

Angiogenesis is a critical process in the human endometrium and ensures that vascular remodelling of the spiral arterioles and regeneration of the vasculature following menstruation can take place. A major angiogenic stimulus is VEGF and it is a member

of the vascular endothelial growth factor family, of which there are six members. VEGF protein and mRNA have been localised within the endometrium across all phases of the menstrual cycle in both the glandular epithelial cells and in the stroma (Charnock-Jones *et al.,* 1993). In cultures of human ESCs progesterone treatment resulted in a rise in VEGF, isoform $VEGF_{189}$, secretion (Ancelin *et al.,* 2001). Hypoxia and cAMP have also been identified as stimuli for VEGF production in ESCs *in vitro* as demonstrated by ELISAs and Northern Blot analysis (Popovici *et al.,* 1999). uNK cells are present in the stroma across the menstrual cycle and increase in number across the secretory phase and into the first trimester of gestation (Loke and King, 1997). Across the secretory phase uNK cells have been shown to express VEGF-C and placenta growth factor (PIGF) mRNA (Li *et al.,* 2001). Additionally, in the late secretoy phase uNK cells express mRNA for the angiopoietin, Ang2. This study also demonstrated the ability of IL-15 to up-regulate VEGF-C mRNA levels in uNK cells. The specific locality of uNK cells within the stroma is in close proximity to the glands and the blood vessels and therefore their production of angiogenic factors may be having a direct influence on the blood vessels. This supports a possible involvement in menstruation or in the stabilisation of blood vessels during implantation and early pregnancy (King and Loke, 1990).

1.2.3 Glandular Cells in Immune and Reproductive Functions

The epithelium is considered central in intestinal immune function (Gewirtz *et al.*, 2002) and in the control of lymphocyte trafficking in, for example, the skin and small intestine (Kunkel *et al.*, 2002). Within the endometrium the glands are composed of epithelial cells and they undergo cyclical variation as described in sections 1.1.1 and 1.1.2. Figure 1.3 illustrates the structure of a gland in the early secretory phase. Infiltrating leukocytes in the mid and late secretory phase are situated immediately adjacent to glands in the human endometrium (Bulmer *et al.*, 1985). Macrophages send long processes into the epithelium of the glands of human endometrium (Kamat *et al.*, 1987) and intraepithelial T-lymphocytes have been identified (Loke *et al.*, 1995). The glandular cells are also HLA-DR-positive in the secretory phase but not in the

proliferative phase (Chiang *et al.*, 1997) and an increased accumulation of extracellular immunoglobulin is apparent within glands with progression of the menstrual cycle (Bjercke *et al.*, 1993) implying a specific immune function in secretory endometrium. Lymphocytes have been characterised in early human decidua and located as either clusters, forming aggregates, near to the glands or as intraepithelial lymphocytes within the glands themselves (Mincheva-Nilsson *et al.*, 1994).

In addition to the leukocyte populations, endometrial epithelial cells are believed to be important in host defence. Defensins are small cationic proteins that confer anti-bacterial, anti-fungal and anti-viral properties and human beta-defensins 1-4 (HBD1-4) mRNA is expressed in the human endometrium (Valore *et al.*, 1998; Fleming *et al.*, 2003; King *et al.*, 2003). Secretory leukocyte protease inhibitor (SLPI) is also a product of human endometrial epithelial cells (King *et al.*, 2000) and has been reported to have antibacterial, antiviral and antifungal effects (Tomee *et al.*, 1998). Both HBD-1 and SLPI increase in the mid secretory phase although SLPI expression rises further in the late secretory phase and into pregnancy (King *et al.*, 2000; Fleming *et al.*, 2003). They may have important functions at the time of implantation and in early pregnancy but since the glands lack the progesterone receptor at this time, stromal-derived factors may be important in controlling their production since the stroma in the functional layer retains the PR-A receptor (Wang *et al.*, 1998).

The COX enzymes are involved in regulation of PGE_2 synthesis and are discussed in section 1.4.1. Both isoforms have been located in the human endometrium with COX-1 expression being predominant in the luminal and glandular epithelial cells (Rees *et al.*, 1982) and COX-2 being expressed in the glands and perivascular cells in the luteal phase (Jones *et al.*, 1997; Marions *et al.*, 1999). Treatment with the antiprogestin, RU486, in the luteal phase reduced the expression of both isoforms in the epithelial cells and a role in endometrial receptivity was proposed (Marions *et al.*, 1999). Studies on mice knock-outs for the COX enzymes have implicated COX-2 in ovulation, fertilisation, implantation and decidualisation (Lim *et al.*, 1997). In the COX-1 knock-

out it has been proposed that COX-2 is compensating for the COX-1 deficiency (Reese *et al.*, 1999).

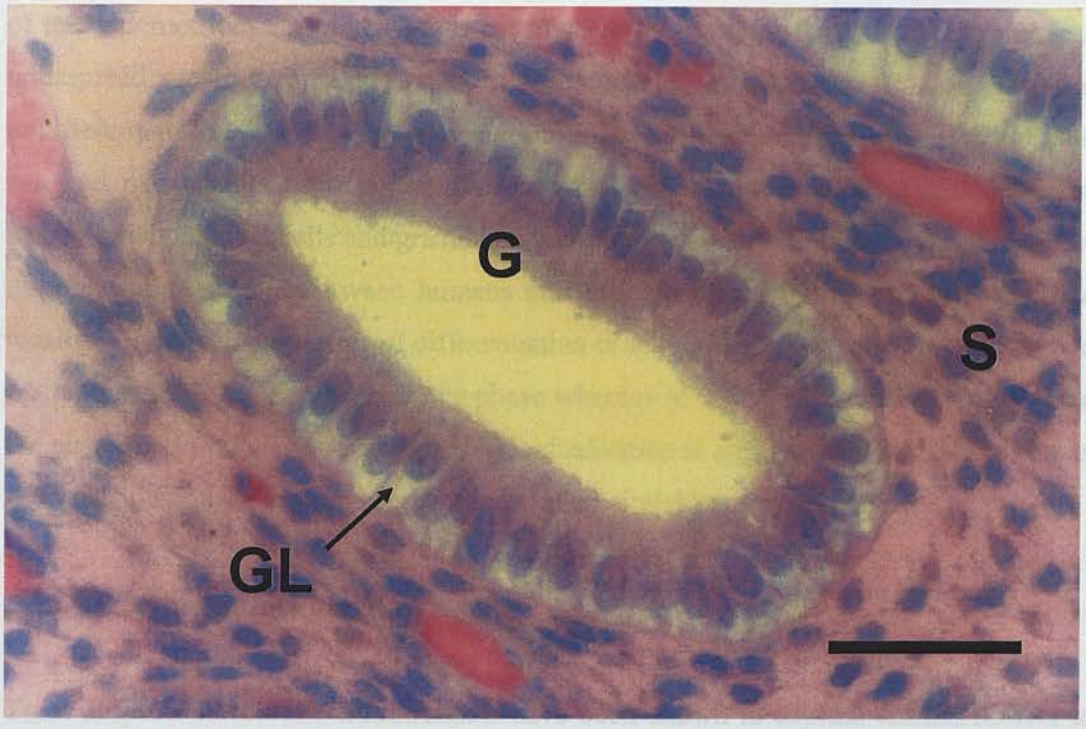
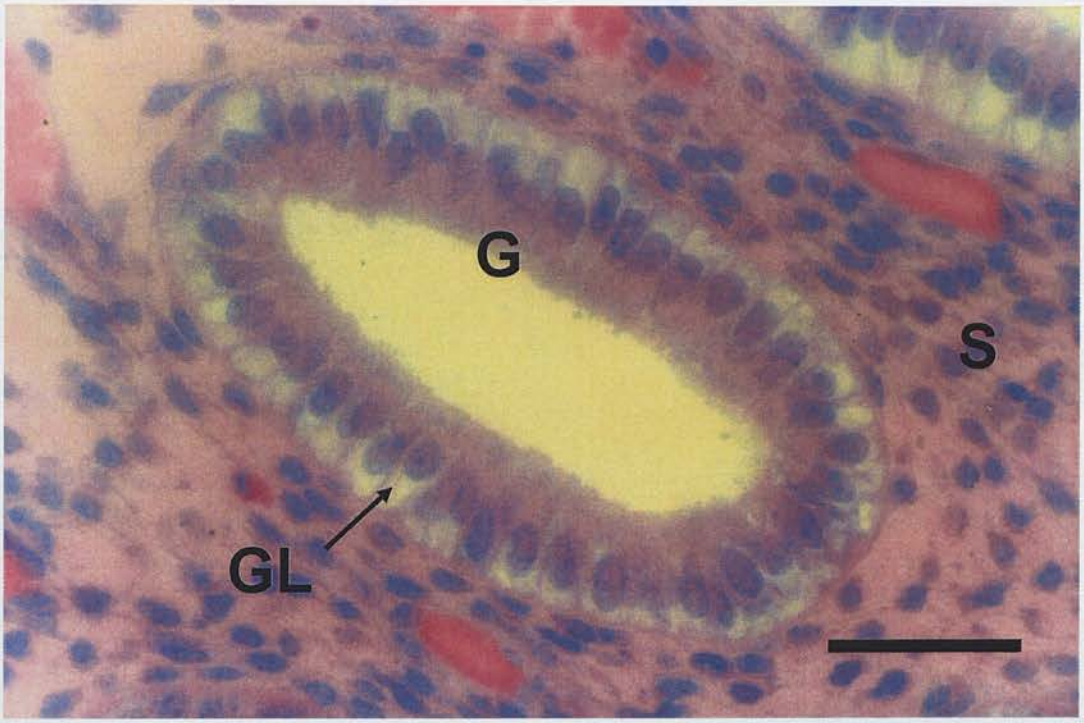


Figure 1.3

Photomicrograph of a gland in the early secretory phase endometrium at high power magnification in human endometrium stained with toluidine blue and acid fuschsin. **G** = gland; **S** = stroma; **GL** = glycogen deposits. Scale bar represents 40μm.



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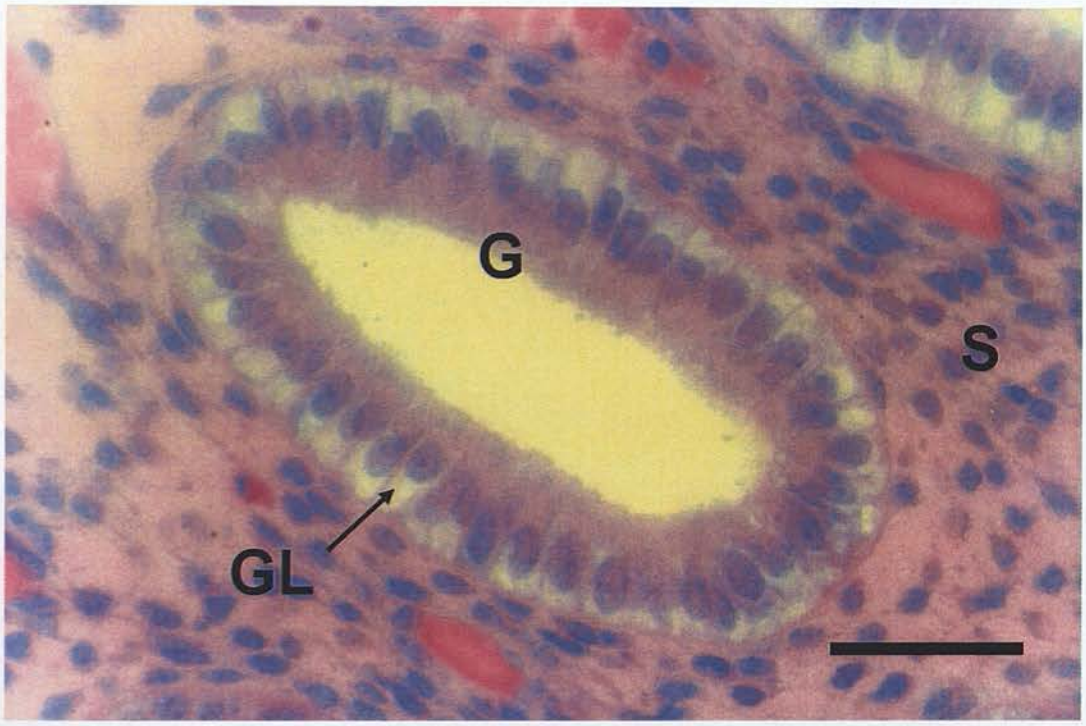


Figure 1.3

Photomicrograph of a gland in the early secretory phase endometrium at high power magnification in human endometrium stained with toluidine blue and acid fuschsin. **G** = gland; **S** = stroma; **GL** = glycogen deposits. Scale bar represents 40μm.

1.3 Establishment of Pregnancy

1.3.1 Decidualisation

The transformation of the human endometrium into decidua begins around post-ovulatory day 9 in oestrogen-primed stromal cells surrounding the spiral arterioles. This reaction then spreads throughout the stroma completing the pre-decidual transformation of the endometrium. If pregnancy is established, these decidual changes become more widespread creating three layers: decidua compacta, decidua spongiosa and a basal undifferentiated layer, allowing regeneration following menstruation or birth. Studies in rats and mice identified three types of decidual cell: anti-mesometrial decidual cells, mesometrial decidual cells and granulated metrial gland cells (De Feo, 1967). However, decidualisation differs between humans and rodents with regard to its stimulus and locality. In humans, the stromal differentiation is spontaneous and occurs in preparation for implantation across the secretory phase whereas in rodents it is initiated only upon the physical stimulus of implantation. Decidualisation is apparent throughout the human endometrium but only occurs at the implantation site(s) in mice and rats.

1.3.1.1 Decidualisation Markers

Term decidual fibroblasts from humans have been shown to express desmin (Oliver *et al.*, 1999). In addition, decidualised ESCs express raised levels of fibronectin and laminin compared with control cultures (Irwin *et al.*, 1989). Rat decidual cells contain large quantities of intermediate fibres. These are made up of vimentin, which is also present in non-decidualised stromal cells, and desmin, specific to decidualised cells (Glasser *et al.*, 1986). However, two other products of the decidual reaction, prolactin and IGFBP-1, are more commonly used to mark this process in culture experiments using human ESCs. In addition to this a further marker of this process is tissue factor and these will be discussed each in turn.

Prolactin is an anterior pituitary hormone, but was first detected being produced outside the pituitary, by decidualised endometrial cells (Riddick *et al.*, 1978). It was then confirmed that prolactin synthesis correlated with the extent of stromal decidualisation in a study of human endometrial explants from the various stages of the menstrual cycle (Maslar *et al.*, 1979). Prolactin is widely expressed and has been located in dermal fibroblasts, epithelial cells of the prostate and breast, Leydig cells of the testis and by certain immune cells as summarised in Ben-Jonathan *et al* 1996 (Ben-Jonathan *et al.*, 1996). Mouse gene knock-outs have confirmed that prolactin is critical to reproductive processes and fertility and particularly with regard to implantation and pregnancy maintenance (Binart *et al.*, 2000). Within the endometrium, prolactin protein is expressed primarily in stromal cells in the late secretory phase but epithelial cells were also immunoreactive for prolactin at this point in the cycle (Bryant-Greenwood *et al.*, 1993). Whilst the protein has also been confirmed in both stromal and epithelial cells, mRNA has only been detected in the stromal cells (Dimitriadis *et al.*, 2000). Prolactin is considered to be important in human implantation and early pregnancy, as discussed in Jabbour and Critchley 2001 (Jabbour *et al.*, 2001). A novel target cell of prolactin has been identified as uNK cells since these cells express the prolactin receptor (Gubbay *et al.*, 2002). Prolactin has also been shown to regulate the expression of interferon-regulatory factor (IRF-1), which is located in the glands and upregulated in the secretory phase (Jabbour *et al.*, 1999).

The insulin-like growth factor system consists of peptide growth factors (IGFs) and their receptors but also specific binding proteins (IGFBPs) that regulate the availability of IGFs to their receptors (Lamson *et al.*, 1991). Six types of soluble IGFBPs exist and these have a degree of tissue specificity (Shimasaki *et al.*, 1991). Within the human endometrium, mRNA for all six IGFBPs were located and with the exception of IGFBP-5, all increased in the secretory phase compared with the proliferative stage (Zhou *et al.*, 1994). The production of IGFBP-2 and -3 by cultures of ESCs with oestrogen and progesterone treatment has been demonstrated (Giudice *et al.*, 1991). Immunohistochemical studies have demonstrated an increase in IGFBP-1 levels in

human endometrium in the secretory phase (Bryant-Greenwood *et al.*, 1993) and IGFBP-1 was found to be a secretory product of secretory phase endometrium and early decidua (Bell *et al.*, 1989). IGFBP-1 is considered a marker of decidualisation and is used to assess this process in cultures of ESCs (Irwin *et al.*, 1989; Bell *et al.*, 1991; Giudice *et al.*, 1992). The specific function of IGFBP-1 in the uterus in unknown although it may be regulating IGF-1, a stimulator of cell growth, and IGF-1 mRNA levels are up-regulated in the rat uterus following oestrogen treatment (Murphy *et al.*, 1987). In women using LNG-IUS mRNA levels of IGFBP-1 are upregulated, consistent with immunohistochemical stromal staining, and IGF-1 levels were found to be decreased (Rutanen, 2000). It was speculated that the inhibitory action of IGFBP-1 on IGF-1 levels could partially account for the progestogenic and anti-oestrogenic effects of LNG-IUS.

Tissue factor is a membrane-bound glycoprotein. It has an extracellular domain that acts as a receptor for factor VII that is involved in the thrombin pathway and it thought to maintain an equilibrium between haemostatic and fibrinolytic pathways as discussed in Schatz *et al* 2001(Schatz *et al.*, 2001). This knowledge, along with *in utero* haemorrhage of tissue factor knockout mice, (Carmeliet *et al.*, 1996) implies a role for tissue factor in regulation of bleeding. In women using Norplant, the observed decline in endometrial tissue factor protein and mRNA levels may partially account for their irregular bleeding patterns (Runic *et al.*, 1997). Tissue factor has also been implicated in angiogenesis via induction of VEGF (Abe *et al.*, 1999). Experiments on human ESCs in culture have confirmed hormonal control over the increase in tissue factor expression and withdrawal of oestrogen and progesterone resulted in a return of tissue factor levels to those similar to pre-treatment levels (Lockwood *et al.*, 1993). Cyclical changes in tissue factor mRNA and protein are observed in humans with greatest levels of expression in luteal phase stroma and in decidua (Lockwood *et al.*, 2001). Its presence may be important in having a primary stabilisation function during the destructive process of trophoblast invasion and paralleled vascular remodelling (Schatz *et al.*, 2001).

1.3.1.2 Progesterone and cAMP

Progestins stimulate the production of prolactin and its receptor in stromal cell primary cultures and this can be inhibited by addition of the anti-progestin, RU486 (Tseng *et al.*, 1999). Studies on ESC decidualisation *in vitro* have demonstrated that progesterone treatment alone is a weak inducer of the decidual transformation and can only achieve this conversion after prolonged exposure (Huang *et al.*, 1987; Zhu *et al.*, 1990; Tseng *et al.*, 1992; Mizuno *et al.*, 1998). It has also been reported that women with low serum progesterone levels at four weeks gestation can have successful pregnancies following assisted reproductive technologies (ART) treatment (Azuma *et al.*, 1993) and it may be the case that other factors are important, in addition to progesterone, to decidualisation. Synthetic progestins, such as medroxyprogesterone acetate (MPA), are usually used in place of progesterone because they are metabolically stable and thus more effective at inducing prolactin expression (Zhu *et al.*, 1990). However, addition of cAMP in combination with progesterone produces this conversion on a greatly reduced time scale (Tang *et al.*, 1993a; Tang *et al.*, 1993b; Brar *et al.*, 1997) and these actions are probably via the protein kinase A pathway (Brar *et al.*, 1997).

1.3.1.3 Relaxin

Relaxin is similar in structure to insulin and insulin-like growth factors (Blundell *et al.*, 1980) and consists of two chains, A and B chains, which are covalently linked by two disulphide bonds (Schwabe *et al.*, 1978; Schwabe *et al.*, 1978). It is synthesised as a precursor form, preprorelaxin (Kemp *et al.*, 1984). Relaxin is primarily a product of the corpus luteum in reproductive tissue but has been isolasted from human decidua by chromatographic elution (Bigazzi *et al.*, 1980). It has been localised to the glands and stroma of human endometrium and decidua, as shown by immunohistochemistry (Bryant-Greenwood *et al.*, 1993). Relaxin is secreted by the ovary and is present in the blood and therefore some of the immunoreactivity for relaxin in the endometrium may be from this source. However, if the sole source of relaxin in this tissue was from

outside of the endometrium it may be expected that highest concentrations of relaxin immunoreactivity would be observed surrounding the blood vessels. In fact, highest immunoreactivity intensity is found in the glandular cells. In addition, two molecular forms of relaxin exist, H1 and H2 and are encoded by different genes (Hudson *et al.*, 1983; Hudson *et al.*, 1984). H2 is the main form produced by the ovary whereas H1 and H2 are produced by decidua (Bryant-Greenwood, 1991; Hansell *et al.*, 1991). Within the stroma, relaxin was immunolocalised in decidualised cells in the late secretory phase and throughout early and late gestation. ESCs themselves have very few receptors for relaxin, approximately 1000 per cell (Osheroff *et al.*, 1995), however, in cultures of human ESCs relaxin both acutely and permanently elevates intracellular cAMP levels in concert with induction of prolactin secretion (Telgmann *et al.*, 1998) and produces maximal stimulus on prolactin production when added in combination with a synthetic progestin (Huang *et al.*, 1987; Zhu *et al.*, 1990). In these early studies porcine relaxin was used in the treatment of human ESCs. Whether this is an exact mimic of human relaxin actions has not been confirmed due to a lack of availability. However, relaxin does have the effect of raising cAMP and the direct application of cAMP to cultures results in prolactin secretion by human ESCs in a similar fashion (Tang *et al.*, 1993a; Tang *et al.*, 1993b; Brar *et al.*, 1997). It has further been demonstrated that the induction of raised cAMP levels by relaxin treatment is due to its action to inhibit phosphodiesterase activity and therefore inhibit cAMP destruction (Bartsch *et al.*, 2001).

1.3.1.4 Interleukin-11 (IL-11)

IL-11 is a member of the gp130 cytokine family along with LIF (Gadient *et al.*, 1999). It has actions in a wide range of environments from haematopoietic cells to the nervous system (Hawley, 1994) but also has anti-inflammatory actions in the gastrointestinal tract (Sands *et al.*, 1999). Within the human endometrium IL-11 is expressed in all cell types with greatest immunostaining intensities apparent in decidualised stroma and appears in these cells before prolactin (Dimitriadis *et al.*, 2000). Human ESCs produce IL-11 and express IL-11 receptor α (IL-11Rα) in culture during progesterone-induced

decidualisation and upon treatment with an anti-human IL-11 antibody a reduction in prolactin and IGFBP-1 levels were observed implicating IL-11 in induction of decidualisation (Dimitriadis *et al.*, 2002). Production of IL-11 by cultured human epithelial cells and stromal cells was enhanced by IL-1α, TNF-α and TGF-β and these results suggest a role for IL-11 in implantation (Cork *et al.*, 2001). In the murine uterus IL-11 is essential to female fertility as shown by IL-11Rα gene knockout studies in which a defective uterine response to blastocyst fails to trigger normal maternal decidual transformation (Bilinski *et al.*, 1998; Robb *et al.*, 1998).

LIF may also have a role in the process of decidualisation since LIF null mice fail to exhibit decidualisation (Stewart *et al.*, 1992b). The reproductive roles of LIF are discussed further in section 1.3.2.2. Recently, mRNA and protein for ghrelin, a peptide hormone, have been shown to increase in human decidualised stroma and in addition, enhanced cAMP-induced decidualisation of cultured human ESCs (Tanaka *et al.*, 2003). The mechanisms and factors involved in decidualisation are complex and it is likely that other factors that are involved have not yet been identified.

1.3.1.5 Decidualisation and Cell Growth

Decidualisation treatments affect the growth rate of ESCs. For example, MPA stimulates cell growth and prolactin production in human ESCs in culture whereas relaxin does not promote cell growth (Zhu *et al.*, 1990). IL-11 increases tritiated thymidine uptake by human ESCs in culture (Karpovich *et al.*, 2003). In rat ESCs both progesterone and prostaglandins have been postulated to exert effects on the rate of DNA synthesis with progesterone stimulating it and PGE_2 reducing it (Peleg, 1983). This is consistent with the finding that in rats treated with RU486, division of stromal cells during pregnancy was inhibited compared with untreated controls (Rider *et al.*, 1994). The proliferative effects of basic fibroblast growth factor (bFGF) are dependent on the presence of progesterone in cultures of human ESCs (Irwin *et al.*, 1991). In addition to this the insulin-like growth factor (IGF) system has been directly implicated

in human ESC decidualisation (Irwin *et al.*, 1994). This study demonstrated the necessity of IGF plus epidermal growth factor (EGF) in combination for promotion of cellular proliferation. However, the presence of growth factors was not essential to stimulate prolactin and IGFBP-1 production. It has been shown that term decidua contains undifferentiated ESCs that can be stimulated *in vitro* to decidualise and it is speculated that these represent a proliferating population of ESCs that are maintained throughout pregnancy but may be recruited to decidualise during gestation (Richards *et al.*, 1995).

1.3.2 Implantation

The main influences on the sequential maturation of the endometrium are oestrogen and progesterone with the latter being dominant in the luteal phase of the cycle. Imbalances of these hormones and/or their receptors can result in an out-of-phase development that may be significantly detrimental to the implantation process (Bonhoff *et al.*, 1990). Studies in rodents demonstrated a maternal implantation window controlled by the steroid hormones (Psychoyos, 1986). In humans, implantation occurs around day LH + 6 and LH + 7 (Hertig *et al.*, 1956; Bergh *et al.*, 1992) and the concept of the "implantation window" specifies that the blastocyst can only implant whilst the endometrium is in a receptive developmental stage (Navot *et al.*, 1989; Li *et al.*, 1991; Tabibzadeh, 1998). The processes of implantation and decidualisation are likely to be closely linked, although in humans decidualisation begins prior to implantation on day LH + 8. However, these two events are likely to be closely coordinated and one important feature of a receptive endometrium is decidualisation. In a review of oocyte donation success, the optimal window for transferring the fertilized oocyte was between cycle days 16 and 19 (Rosenwaks, 1987). After this period, no pregnancies were established. Ultrastructual changes in the luminal epithelial cells are also thought to be correlated with this receptivity status (Sarani *et al.*, 1999). However, implantation has been shown to be dependent on embryonic age rather than endometrial maturation in another study involving IVF treatment of women (Bergh *et al.*, 1992). The receptive endometrium is thought to balance implantation with excessive trophoblast invasion by providing appropriate signals (Tabibzadeh *et al.*, 1995) and embryonic signalling may be essential for the implantation process and maternal acceptance (Edwards, 1994).

The implantation process itself is quite unique in humans in comparison to other species and is haemochorial (Pijnenborg *et al.*, 1981). The first stage of implantation is fertilisation of the oocyte within the fallopian tube. The fertilized oocyte then undergoes successive cleavages during the week-long peri-implantation period. These cleavages must generate sufficient cell numbers prior to blastocyst formation to allow completion

of inner cell mass and trophectoderm formation and are followed by proliferation and migration towards the uterine cavity around 72–96 hours post fertilisation. On day 5, the blastocyst hatches from the zona pellucida. The actual process of implantation occurs in three stages, the first of these being apposition. This is followed by adhesion of the blastocyst to the luminal epithelium via the trophectoderm and allows the trophoblast to burrow between the luminal epithelial cells, leaving these luminal epithelial cells undisturbed. Placenta formation starts once the trophectoderm has invaded and become embedded in the decidua. Some of the cytotrophoblast cells then extend into the peripheral syncytium and join to form the cytotrophoblastic shell. Villous trophoblast cells will eventually form a layer over the placenta and have the function of nutrient and oxygen transport from maternal blood to the fetus. The extravillous trophoblast cells penetrate deep into the decidua and transform the spiral arteries, destroying the muscular walls and converting them into larger vessels allowing delivery of greater volumes of blood. This is essential for an adequate blood supply for the fetus and in cases of under-invasion of the trophoblast pathologies such as miscarriage, pre-eclampsia and fetal intrauterine growth restriction can result (Loke *et al.*, 1995). This is not only relevant to the short-term with regard to pregnancy complications but can lead to the development of problems in adult life (Barker, 1997b). Conversely, when over-invasion of the trophoblast occurs a phenomenon of placenta percreta, or uterine rupture, can develop. Therefore, it is important for this process to be under stringent control and a synchrony between the blastocyst and endometrium is required for it to be implemented successfully.

1.3.2.1 Colony Stimulating Factor-1 (CSF-1)

Signalling between the blastocyst and the endometrium are likely to be essential prior to adhesion to the epithelium and several soluble factors have been implicated. Colony-stimulating-factor-1 (CSF-1) is a growth factor and is expressed within the endometrium in the stroma and epithelial cell and also by the trophoblast along with its receptor, c-fms (Daiter *et al.*, 1992). Mutant mice lacking CSF-1 are infertile due to implantation failure

(Pollard *et al.*, 1991). The cyclical variation of CSF-1 and c-fms in humans is characterised by rapid rises in expression in the late secretory phase and early pregnancy (Kauma *et al.*, 1991).

1.3.2.2 Leukaemia Inhibitory Factor (LIF)

LIF is a glycoprotein expressed in endometrial glands just prior to implantation in mice (Bhatt *et al.*, 1991) and may be involved in blastocyst growth and implantation (Bulletti *et al.*, 1994; Polan *et al.*, 1995). A targeted mutation of the maternal LIF gene resulted in impaired implantation in mice. The blastocysts however were viable and able to implant in wild-type pseudo-pregnant mice implicating LIF specifically in implantation (Stewart *et al.*, 1992b). In contrast, LIF is not critical to implantation in sheep and cows as determined by experiments in which animals were immunised against LIF (Vogiagis *et al.*, 1997). In humans, LIF is present within endometrium and at highest levels during the time of implantation (Charnock-Jones *et al.*, 1994; Kojima *et al.*, 1994). Isolated first trimester trophoblast is negative for LIF but positive for its receptor (King *et al.*, 1995) and it could be speculated that endometrial LIF is acting on its receptors located on trophoblast cells. Some of the actions of LIF may be via human chorionic gonadotrophin (hCG) since anti-hCG antibodies have been shown to block LIF-mediated trophoblast differentiation (Sawai *et al.*, 1995). hCG is secreted by the embryo and by preventing demise of the CL and therefore maintains luteal progesterone production. The actions of hCG may not be confined to the CL since hCG receptors have also been located in the human endometrium (Reshef *et al.*, 1990) and in support of this, human endometrial stromal cells treated with hCG decidualise (Han *et al.*, 1999).

1.3.2.3 Cell-matrix Interactions

Interactions between the extracellular matrix and cells provide an essential communication route. The interactions between extracellular matrix constituents and the surface of cells are predominantly mediated by integrins. Integrins are a family of cell surface heterodimeric α/β glycoproteins and are able to bind matrix molecules and to cell surface receptors (Hynes, 1992). They participate in adhesion, migration, proliferation, differentiation and cell survival. Several integrins are expressed within the human endometrium (Lessey *et al.*, 1992; Tabibzadeh, 1992). The expression of many of these fluctuates in a cyclical manner and those with expression confined to the window of implantation are $\alpha_1\beta_1$, $\alpha_4\beta_1$ and $\alpha_v\beta_3$ (Tabibzadeh, 1992; Lessey *et al.*, 1994a). Expression of the fibronectin receptor is delayed in luteal phase defect (Lessey *et al.*, 1992) and aberrant expression of this receptor has also been associated with endometriosis (Lessey *et al.*, 1994b).

Matrix metalloproteinases (MMPs) are enzymes that have a range of specificity for extracellular matrix molecules and are invloved in their degradation. MMPs are generally considered pathological when detected in connective tissue, however, they have been localised in human trophoblast (Polette *et al.*, 1994) and are considered to have a role in the uterus of physiological tissue remodelling (Hulboy *et al.*, 1997). Tissue inhibitor of MMPs (TIMP) inhibits the invasiveness of trophoblastic cells *in situ* (Graham *et al.*, 1991). MMP2 and MMP9 are members of the gelatinase subfamily and are secreted by the invasive human trophoblast (Shimonovitz *et al.*, 1994). They act to digest collagen type IV, the major constituent of the uterine basement membrane, and would be important in uterine invasion during implantation. MMPs are discussed in more depth in section 1.1.4.2.

The interactions between leukocytes and ESCs via the production of cytokines and growth factors, such as VEGF, the interferons and TNF-α, are believed to be important in endometrial tissue breakdown and remodelling (Fraser, 1999). These immune cells

are believed to play a significant role in establishment of microenvironments via their production of such factors (Tabibzadeh *et al.,* 1993; King, 2000). uNK cells are located in the stroma in high concentrations around the blood vessels and are a source of VEGF and their potential effects on the blood vessels are briefly assessed in section 1.2.2.2. In addition, uNK cells are also located at the implantation site and this is their only location within the mouse endometrium (Croy *et al.,* 1991). They may be involved in modulating trophoblast invasion in humans (King and Loke, 1990) and in mice have been proposed as having a role in the regulation of placental development whereby knockout mice for uNK cells exhibit reduced placental size (Croy *et al.,* 2002). However, a more crtical role in mice is the stabilisation of the vasculature and complete differentiation of the endometrium into decidua (Ashkar *et al.,* 2000). IFN-γ-producing immune cells, in particular uNK cells, are thought to be involved in modulating ESC decidualisation in humans (Christian *et al.,* 2001). They demonstrated the ability of IFN-γ to antagonise prolactin protein and mRNA expression in primary ESC cultures. This provides a role for IFN- γ as a modifier of ESC function.

1.3.3 The Role of Cytokines

Cytokines are small glycoproteins with autocrine and paracrine interactions and are predominantly associated with immune functions. They can be involved in both the induction and resolution of an inflammatory response and can generally be termed "pro-inflammatory" or "anti-inflammatory". Studies on CD4+ T-cells showed that responses of these cells are polarised and they have been classified on their cytokine production as either T-helper-1 (Th-1) or T-helper-2 (Th-2) (Mosmann *et al.*, 1986). The Th-1 cytokines include IFN-γ, IL-2 and TNFα and whilst examples of Th-2 cytokines are IL-4, IL-10 and IL-13. In the human endometrium during the peri-implantation phase, a reduction or absence of Th-1 and a dominance of Th-2 cytokines is observed (Lim *et al.*, 1998). The general hypothesis with regard to pregnancy that has been derived from mice studies is that a Th-1 response is considered detrimental and a Th-2 response is thought to be beneficial and supportive of pregnancy and the balance of these responses

is likely to be critical (Wegmann *et al.*, 1993; Hill *et al.*, 2000), although it is now apparent that this is an over-simplification (Chaouat *et al.*, 2002). Some of the reproductive functions of cytokines are summarised in table 1.2.

Cytokine	Endometrial function
IL-1	Induction of adhesion molecules Modulation of proliferation Alteration of morphology Induction of cytokines Chemotaxis and induction of lymphoid infiltration Oedema Induction of PGE_2 Activation of T cells
TGF-β	Gland formation and Angiogenesis
IFN-γ	Induction of adhesion molecules Modulation of proliferation Alteration of morphology Induction of cytokines Chemotaxis and induction of lymphoid infiltration Induction of menstruation Induction of HLA-DR and ICAM-1
TNF-α	Induction of adhesion molecules Modulation of proliferation Alteration of morphology Induction of cytokines Chemotaxis and induction of lymphoid infiltration Oedema Induction of PGE_2 Injury to endometrial vessels Activation of polymorphonuclear leukocytes

Table 1.2

Cytokine functions in the human endometrium. Table adapted from Tabibzadeh 1994 (Tabibzadeh, 1994a).

This section will examine IL-1, IL-10 and IFN-γ and their functions within the uterus. IL-8 and IL-15 are discussed in more detail in sections 1.2.1.2 and 1.4.3 respectively.

1.3.3.1 Interleukin-1 (IL-1)

IL-1 is a multifunctional cytokine and exists in two forms: IL-1α and IL-1β with a low sequence homology between them. They have similar action and act on the same receptor, IL-1 type-1. A second receptor sub-type exists, IL-1 type-2, but it is non-functional. In humans, the IL-1 receptor antagonist is located in decidual glands, and in isolated cells in chorionic villi, the intervillous space and in maternal decidua (Simon *et al.*, 1994). These decidual cells staining positive for the IL-1 receptor antagonist are thought to be macrophages (Tabibzadeh *et al.*, 1992). IL-1β is the dominant form and is secreted by activated murine peritoneal macrophages (Chensue *et al.*, 1989). In the human endometrium both IL-1α and IL-β are expressed in the epithelial and stromal cells (Tabibzadeh *et al.*, 1992) and IL-β mRNA expression is raised in pregnancy (Kauma *et al.*, 1990). Human serum IL-1 levels vary according to the menstrual cycle and are found to be highest in the secretory phase, post-ovulation (Cannon *et al.*, 1985). In contrast, IL-1 receptor antagonist levels decline in the secretory phase (Simon *et al.*, 1995). IL-1 has also been connected to blastocyst implantation and is found expressed along with its receptor in both trophoblast and decidual cells (Simon *et al.*, 1994; Simon *et al.*, 1995). ESCs produce an immune-related response upon treatment with IL-1α by evoking an enhancement in phagocytosis of latex particles and E.coli (Ruiz *et al.*, 1997). This cytokine may also be involved in menstruation since in cultures of human ESCs, IL-1α stimulates production of MMP-1, a key enzyme in menstruation initiation (Singer *et al.*, 1997). IL-1β acts to raise PGE_2 levels in decidual and endometrial ESCs (Cole *et al.*, 1995; Ishihara *et al.*, 1995). IL-1α stimulates PGE_2 and $PGF_{2\alpha}$ production in ESCs (Kawano *et al.*, 2001). These data support a role for IL-1 in the regulation of menstruation since PGE_2 and $PGF_{2\alpha}$ are believed to be involved in vascular changes associated with endometrial tissue degradation (Baird *et al.*, 1996). IL-1 has also been

study was also assessing NK cells from blood and whether they have any impact on uNK cells has not been confirmed.

1.3.3.3 Interferon-γ (IFN-γ)

The interferon family are cytokine mediators that are involved in alterting the immune system to the presence of viral infections. The family comprises of three members in humans: IFN-α, -β and -γ. Components of the same receptor are shared by IFN-α and IFN-β and they are therefore referred to as type I interferons. Since IFN-γ uses a separate receptor system it is termed a type II interferon. In addition to distinct receptors, type I and II interferons also differ with regard to which chromosome they are located on and their structure differs. IFN-γ is distinct due to its sensitivity to pH and temperature extremes (Arai *et al.*, 1990) and is expressed by many cell types including Natural Killer cells and macrophages, but predominantly by T cells (Sugawara *et al.*, 1986). In contrast, type I interferons are secreted by virus-infected cells (Le Page *et al.*, 2000).

IFN-τ is a member of the interferon I family. It is not present in humans but is expressed by ruminant trophoblastic cells although it has structural and biological functions similar to those of IFN-α and -β, both present in humans (Roberts *et al.*, 1999). IFN-τ is believed to be the maternal recognition of pregnancy signals in ruminants. IFN-τ is also secreted by the bovine conceptus and is considered to be involved in rescue of the corpus luteum via limitation of $PGF_{2\alpha}$ release (Thatcher *et al.*, 1995).

The source of IFN-γ within the human endometrium is thought to be the lymphoid aggregates (Tabibzadeh, 1994b) but is also synthesised by uNK cells in first trimester decidua (Saito *et al.*, 1993) and by macrophages (Gessani and Belardelli, 1998). IL-12 can stimulate IFN-γ production in cultures of uNK cells and acts synergistically with IL-

2 to provide this response (Marzusch *et al.*, 1997). IFN-γ levels remain consistent across the menstrual cycle in non-pregnant endometrium (Yeaman *et al.*, 1998) but during pregnancy it can only be detected in supernatants from first trimester decidua and not in the later pregnancy stages (Lin *et al.*, 1993). IFN-γ acts on the epithelial cells to induce human leukocyte antigen (HLA)-DR molecules of MHC and triggers morphological changes in addition to a reduction in growth (Tabibzadeh *et al.*, 1986; Tabibzadeh *et al.*, 1988). This cytokine also has actions on ESCs to induce an increase in the levels of IL-6, MCP-1 and MCSF production and reduce IL-8 levels (Nasu *et al.*, 1998).

Table 1.3

Summary of the reproductive phenotypes exhibited by mutated mice genetically deficient in endometrial cytokines or their receptor. This table is adapted from Robertson and Hudson 2002 (Robertson *et al.*, 2002).

Cytokine	Mutated reproductive phenotype	Reference
CSF-1	Macrophage deficiency Gametogenesis dysregulation	Pollard *et al* 1991 (Pollard *et al.*, 1991)
GM-CSF	Increased fetal reabsorption Placental abnormalities	Seymour *et al* 1997 (Seymour *et al.*, 1997) Robertson *et al* 1999 (Robertson *et al.*, 1999)
IFN-γ	Dysregulation of uNK cells Decidual necrosis Inadequate transformation of endometrial vessels during decidualisation Increased fetal reabsorption	Ashkar and Croy 1999 (Ashkar *et al.*, 1999)
IL-1R	Reduced litter size	Abbondanzo *et al* 1996 (Abbondanzo *et al.*, 1996)
IL-5	Eosinophil deficiency Apparently uncomplicated pregnancy	Robertson *et al* 2000 (Robertson *et al.*, 2000)
IL-11Rα	Defective decidualisation Failed implantation	Robb *et al* 1998 (Robb *et al.*, 1998)
IL-2 Rγ	Fail to develop uNK cells	Miyazaki *et al* 2002 (Miyazaki *et al.*, 2002)
LIF and its R	Failed implantation Placental abnormalities	Stewart *et al* 1992 (Stewart *et al.*, 1992b) Ware *et al* 1995 (Ware *et al.*, 1995)
TGF-β1	Lethal in embryogenesis	Shull and Doetschman 1994 (Shull *et al.*, 1994)

1.3.3.4 The mouse as a model

In order to evaluate the relevance of different pathways and compounds to an overall system the mouse proves to be a versatile tool to use. In this respect, knockout mice offer exploration to the function of specific genes and their products whereby such an in depth study cannot be carried out on humans. Examples of mouse knockouts that are relevant to reproductive processes are summarised in table 1.3. Although these knockouts give an insight into a physiological situation, these findings in mice cannot be directly applied to the human situation. With regard to the uterus there are many differences between the mouse and the human. The process of decidualisation in humans occurs in anticipation of pregnancy whereas in mice the physical stimulus of implantation is the trigger. This implies that the control of this process in humans is divergent to that in mice. In addition, decidualisation only occurs at the sites of implantation in mice, which is highly specific compared to the dispersed decidual transformation within the human endometrium. Therefore to what extent, for example, IL-11 is essential to decidualisation and implantation in humans may not directly correlate to that occurring in mice (Robb *et al.*, 1998). However, studies using cultures of human ESCs have also indicated IL-11 having a role in decidualisation (Cork *et al.*, 2001; Dimitriadis *et al.*, 2002) therefore illustrating the use of the mouse model as starting place. In the case of the COX-1 knock-out mouse it appears that COX-2 is compensating for the absence of COX-1 and therefore the exact roles of this enzyme become unclear (Reese *et al.*, 1999). This could be occuring in other mouse knock-outs and therefore should be taken into consideration when assessing the results.

With regard to uNK cells the mouse has been used as a model to assess their function in several studies (Ashkar and Croy, 1999; Ashkar *et al.*, 2000; Croy *et al.*, 1991; Croy *et al.*, 2002; Zhang *et al.*, 2003). These studies have focused on knock-out models in particular and the possible functions of uNK cells in humans are discussed in section 1.2.2. However, more recently IL-15 knock-out mice, which are deficient in uNK cells, have demonstrated that these cells are not essential to pregnancy (Barber and Pollard,

2003; Ashkar *et al.*, 2003). What these studies did show was that birth weight was compromised, probably the result of inadequate conversion of the uterine vasculature, (Barber and Pollard, 2003) and this deficiency in weight continued into adulthood (Ashkar *et al.*, 2003). This could have implications on coronary function in the longterm. The work of Barker demonstrated a greater incidence of coronary heart disease and stroke in individuals who had suffered from retarded fetal growth (Barker, 1997a, b and c). In humans the uNK cell may be of greater relevance to the establishment and maintenance of pregnancy than in mice but it may also have implications to intrauterine programming due to its role in blood vessel modification. The distribution of uNK cells is different between mice and humans with them only located at the metrial triangle in mice whereas in humans they are distributed throughout the stroma. These uNK cell knock-out mice do exhibit defective decidualisation (Ashkar *et al.*, 2000) and since this decidual transformation is a pre-requisite to implantation in humans this lack of uNK cells may have a more significant contribution to establishment of pregnancy in this species.

As discussed in Enders, 2000, the mouse proves an economic animal to use as a model, however, differences including the mechanism of implantation and the shape of the lumen exist (Enders, 2000). The mouse is a useful tool and provides a rational starting point for the exploration within the human system or within other animal models more closely related to humans.

1.4 Molecular Contributions to Pregnancy

1.4.1 The Prostaglandin Cascade

Prostaglandin (PG) E_2 and $PGF_{2\alpha}$ are the most abundant PGs in the human endometrium. The major site of production is the glandular epithelial cells both in the endometrium and in early pregnancy (Smith *et al.*, 1988). The production of prostaglandins (PGs) is controlled by two rate-limiting steps, phospholipase A_2 activity

and activity of the two cyclooxygenase enzymes, COX-1 and -2 (figure 1.4). Initially, arachidonic acid (AA) must be liberated from the phospholipid membrane of the cell via PLA_2. This free AA can then be converted to prostaglandin H_2 (PGH_2) by COX-1 and –2. The next stage in the cascade is the action of specific synthases on PGH_2 to direct the production of the five primary prostanoids, namely PGD_2, PGE_2, $PGF_{2\alpha}$ PGI_2 and TXA_2 (Smith *et al.*, 1996; Smith *et al.*, 1996). Three PGE synthases (PGES) have been identified to date, and these include a cytosolic PGES (cPGES) (Tanioka *et al.*, 2000) and two microsomal PGESs, mPGES-1 (Murakami *et al.*, 2000; Mancini *et al.*, 2001) and mPGES-2 (Tanikawa *et al.*, 2002). Aberrant expression of mPGES-1 in conjunction with COX-2 has been postulated to have a role in tumourigenesis (Kamei *et al.*, 2003). In reproductive tissue, PGES has been located in bovine endometrium and its expression correlated with that of COX-2 (Parent *et al.*, 2002). Studies in mice have demonstrated the high expression of mPGES in the stroma that immediately surrounds the blastocyst and have implied a role for this synthase in decidualisation and implantation in rodents (Ni *et al.*, 2002). Conversely, PGs are metabolised and thus deactivated by prostaglandin dehydrogenase (PGDH) enzymes. In the uterus, PGDH has been speculated to have a role in maintaining low concentrations of PGs in the fetal membranes during the majority of pregnancy (Cheung *et al.*, 1992). In some cases of idiopathic preterm labour, a reduction in PGDH expression in the chorionic trophoblast resulting in reduced PG regulation, has been implicated as a causative factor (Sangha *et al.*, 1994). In support of a role for PGs in pre-term labour, inhibition of COX-2 can prevent inflammation-mediated pre-term labour in mice (Gross *et al.*, 2000b). Within the human endometrium, PGDH is located within the cytoplasm of gland cells and levels were found to be highest in the secretory phase but lowest pre-menstrually and during menstruation (Casey *et al.*, 1980). Raised levels in the secretory phase versus the proliferative phase were confirmed in a later study and it was also demonstrated that PGDH activity was significantly increased at 12 months post-insertion of a LNG-IUS and coincided with a rise in PR-A and –B immunostaining (Critchley *et al.*, 1998b). This correlates with the control of endometrial bleeding, implicating both receptor levels and PGs in the process. The role of PGs is supported additionally by a further study that

has demonstrated a decline in PGDH immunostaining in the glands and stroma in women 36-48 hours after receiving the antiprogestin, mifepristone (Hapangama *et al.*, 2002).

PGs have a short half-life and are metabolised rapidly. It is therefore likely that their actions are local to their production site. PGs are classified by their cyclopentane ring and TXA contains an oxane ring. Each group acts via its own distinct receptors (Coleman *et al.*, 1994; Narumiya *et al.*, 1999). These receptors are G protein-coupled receptors with 7 transmembrane domains, located on the plasma membrane. Sequence homology between the different receptors is low, ranging from 20 to 30%, although homology of the specific receptors between species is far higher, 76 – 97% (Narumiya *et al.*, 1999). There are 4 different PGE_2 receptors and these are referred to as EP_{1-4}. The EP_2 and EP_4 receptors act via G_s proteins and have the effect of raising cAMP levels. The EP_3 receptor exists as 7 splice variants (Adam *et al.*, 1994; Sugimoto *et al.*, 2000) with the predominant effect of decreasing cAMP levels. EP_1 acts via a different second messenger, Ca^{2+}, to exert its effects (Kennedy *et al.*, 1982; Watabe *et al.*, 1993).

COX-1 is constitutively expressed in most tissues and in contrast COX-2 is normally absent and only present in a pathological state, for example, COX-2-dependent pathways have been implicated in development of colorectal cancers (Williams *et al.*, 1999) and in eutopic and ectopic pregnancies (Ota *et al.*, 2001). COX-2 can be induced by factors such as lipopolysaccharide (Inoue *et al.*, 1995) and IL-1 (Thomas *et al.*, 2000; Tamura *et al.*, 2002). However, it is becoming apparent that COX-1 can also be induced and it has been shown to be upregulated in cervical carcinomas (Sales *et al.*, 2002). Both of the COX isoforms are inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) and it has been demonstrated in mice and humans that some NSAIDs are preferentially selective for one or other of the COX enzymes (Meade *et al.*, 1993; Gierse *et al.*, 1995). The cause of luteinised unruptured follicle from animal and clinical studies may be related to COX-2 inhibition and a link between NSAIDs and reversible female infertility has been proposed (Stone *et al.*, 2002). In the human endometrium around the time of

implantation, COX-1 and COX-2 are expressed in glandular and luminal cells, and in the luminal and perivascular cells respectively (Marions *et al.*, 1999). When mifepristone was administered, epithelial immunostaining of COX-1 and –2 declined. In addition to illustrating a connection between the PG pathway and steroid hormone action, a function in endometrial receptivity has been suggested (Marions *et al.*, 1999). COX-2 immunoreactivity levels are greater in the premenstrual phase than the mid-secretory phase and this coincides with the recruitment of leukocytes into the endometrium immediately prior to menstruation (Jones *et al.*, 1997).

A variety of PG antagonists have been shown to reduce the implantation site number in mice and it has been suggested that PGs exert effects on both mother and fetus (Biggers *et al.*, 1981). Many studies have assessed the effects of the PG pathway on female fertility and reproductive processes by generating gene knockouts for the various aspects of the cascade. Some of these will be discussed here although it is important to remember that reproductive events such as decidualisation and parturition (Gross *et al.*, 2000a) differ between mice and humans. A double knock-out for COX-1 and COX-2 is lethal, caused by patent ductus arteriosus (Loftin *et al.*, 2001), and therefore it is not possible to study the reproductive consequences in mice of complete COX absence. Targeted disruption of COX-2 in mice leads to problems with the key processes of ovulation, fertilization, implantation and decidualisation (Lim *et al.*, 1997). Studies in mice show two distinct pathways for COX-1 and –2 within the uterus implying independent contributions to uterine PG production but these were also shown to overlap to some extent (Reese *et al.*, 2001). In a previous study on knock-out mice for COX-1 (-/-), on pregnancy day 4 demonstrate reductions in vascular permeability and PG concentration (Reese *et al.*, 1999). However, these reductions were less than those predicted and further investigation revealed a compensatory response by COX-2 demonstrating interaction between the two enzymes. Mice deficient in the EP_2 receptor exhibit a reduced ovulatory number and a reduced fertility rate (Hizaki *et al.*, 1999). No alterations in uterine development are apparent. The lack of EP_4 receptor expression in mice knockouts proves to be fatal soon after birth due to malfunction of the ductus

arteriosus. Mice deficient in cytosolic PLA_2 have reduced fertility, smaller litter sizes and delayed initiation of labour (Bonventre *et al.*, 1997; Uozumi *et al.*, 1997).

1.4.1.1 PGE_2 as an Immune Modulator

PGs have been implicated as modulators of immunity (Harris *et al.*, 2002) and in support of this PGE_2 can inhibit many immune cells such as T cells (Goodwin *et al.*, 1983) and neutrophils (Fantone *et al.*, 1983). Within human semen high concentrations of PGE_2 are contained and this is believed to aid reproductive success via inhibition of female immune responses (Kelly, 1991). Decidual stromal cells are a source of PGE_2 and this PG has been shown to have an inhibitory effect on NK cells via the IL-2 and IL-15 receptors (Parhar *et al.*, 1989; Joshi *et al.*, 2001). Addition of indomethacin or anti-PGE_2 antibody to NK cells *in situ* revealed an up-regulation of the IL-2 receptor α chain, IL-2 production and an increased anti-trophoblast killer activity (Parhar *et al.*, 1989). PGE_2 also has the ability to alter the cytokine profile of cells and favour a Th-2 response in preference to a Th-1 reaction by stimulating IL-10 and inhibiting IL-12 production in blood monocytes (van der Pouw Kraan *et al.*, 1995).

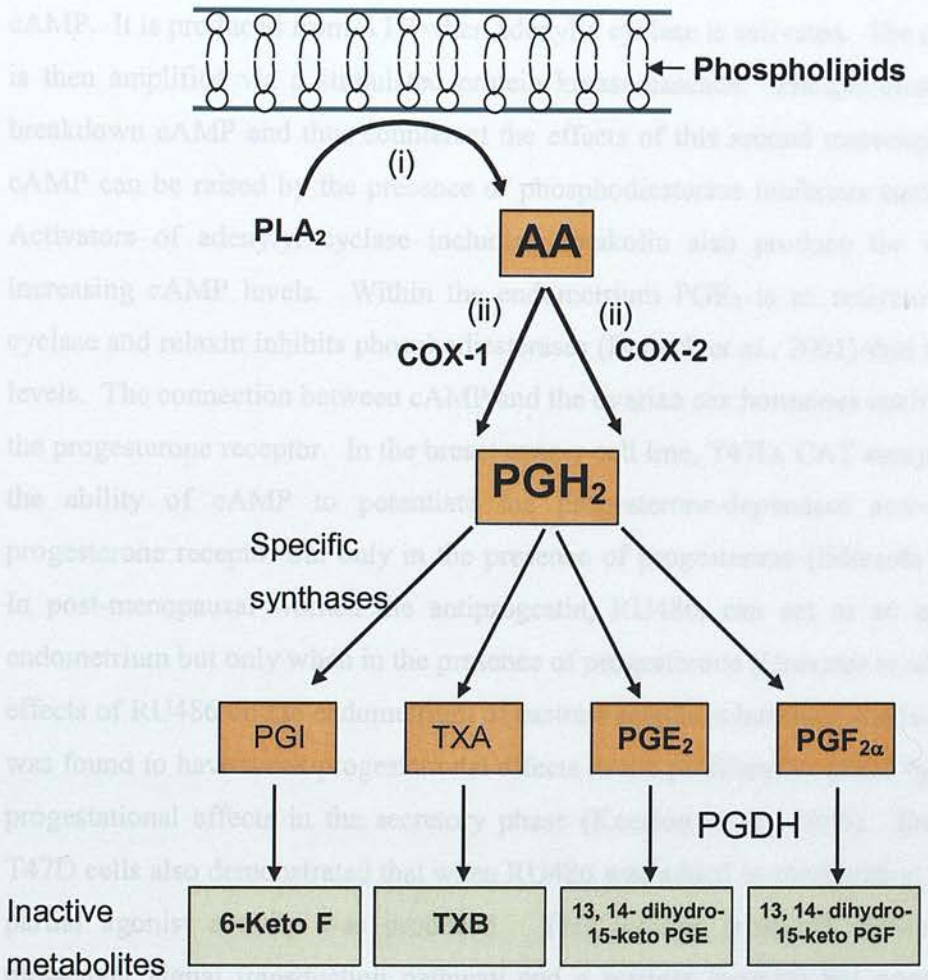


Figure 1.4

The prostaglandin cascade illustrating the two rate-limiting stages (i) PLA_2 activity to liberate free arachidonic acid (AA) from membrane phospholipids and (ii) COX-1/-2 activity in the conversion of AA to PGH_2. The PGs and TXAs then act on their specific GPCRs.

1.4.2 Cyclic Adenosine Monophosphate (cAMP)

Intracellular second messengers allow the transmission of signals to pass within a cell and produce an amplification of the signal. One of these important messengers is cAMP. It is produced from ATP when adenylyl cyclase is activated. The original signal is then amplified via a stimulated protein kinase cascade. Phosphodiesterases act to breakdown cAMP and thus counteract the effects of this second messenger. Levels of cAMP can be raised by the presence of phosphodiesterase inhibitors such as rolipram. Activators of adenylyl cyclase including forskolin also produce the end result of increasing cAMP levels. Within the endometrium PGE_2 is an activator of adenylyl cyclase and relaxin inhibits phosphodiesterases (Bartsch *et al.*, 2001) thus raising cAMP levels. The connection between cAMP and the ovarian sex hormones could be partly via the progesterone receptor. In the breast cancer cell line, T47D, CAT assays have shown the ability of cAMP to potentiate the progesterone-dependent activation of the progesterone receptor but only in the presence of progesterone (Edwards *et al.*, 1993). In post-menopausal women the antiprogestin, RU486, can act as an agonist in the endometrium but only when in the presence of progesterone (Gravanis *et al.*, 1985). The effects of RU486 on the endometrium of castrate monkeys has been studied, and RU486 was found to have weak progestational effects in the proliferative phase but strong anti-progestational effects in the secretory phase (Koering *et al.*, 1986). Experiments on T47D cells also demonstrated that when RU486 was added in combination with cAMP a partial agonist activity was produced. This implies cross-talk between a second messenger signal transduction pathway and a nuclear receptor but whether this also occurs within the endometrium has yet to be established.

Within the human endometrium, cAMP is the main second messenger involved in the PGE_2 signal transduction pathway (Frank *et al.*, 1994). Evidence for the involvement of cAMP and its analogues in the decidualisation of human endometrium has been proposed (Tang *et al.*, 1993a; Brosens *et al.*, 1996; Brar *et al.*, 1997; Brosens *et al.*, 1999). Experiments using cultures of ESCs have shown that prolactin expression is

dependent on activation of the PKA pathway (Brar *et al.*, 1997; Telgmann *et al.*, 1997). Direct use of cAMP results in an increase in prolactin mRNA expression and protein release (Tang *et al.*, 1993a; Brosens *et al.*, 1996; Brar *et al.*, 1997; Brosens *et al.*, 1999) and this elevation is a permanent response (Telgmann *et al.*, 1998). They demonstrated the ability of cAMP to synergise with a synthetic progestin to result in stromal differentiation and an increase in prolactin levels. Progesterone treatment alone proved to be a weak inducer of the decidualised phenotype. High intracellular cAMP levels appear essential by sensitising the cells to progestins and thus providing maximal prolactin expression. Deletion of the region between position –332 and –270 in the dPrl promoter caused a strong reduction in the ability of cAMP to induce prolactin expression implying the importance of this particular region in its activation (Telgmann *et al.*, 1998).

1.4.3 Interleukin-15 (IL-15)

IL-15 was initially discovered as a stimulator of T-cell proliferation (Grabstein *et al.*, 1994). It has a molecular weight of 14-15 kDa and is a member of the 4 α-helix cytokine family. The receptor complex for IL-15 is composed of three subunits. The α-chain is unique to its receptor but both the β- and γ-chains are also common to the IL-2 receptor (Carson *et al.*, 1994; Grabstein *et al.*, 1994). Two forms of IL-15 exist due to alternative splicing resulting in a long signal peptide (LSP), 48-AA in length, and a short signal peptide (SSP), 21-AA long (Fehniger *et al.*, 2001) (figure 1.5). The SSP IL-15 is restricted to the nuclear and cytoplasmic compartments of the cell whereas the LSP has been detected in the golgi apparatus and is thought to follow a secretory route (Tagaya *et al.*, 1997).

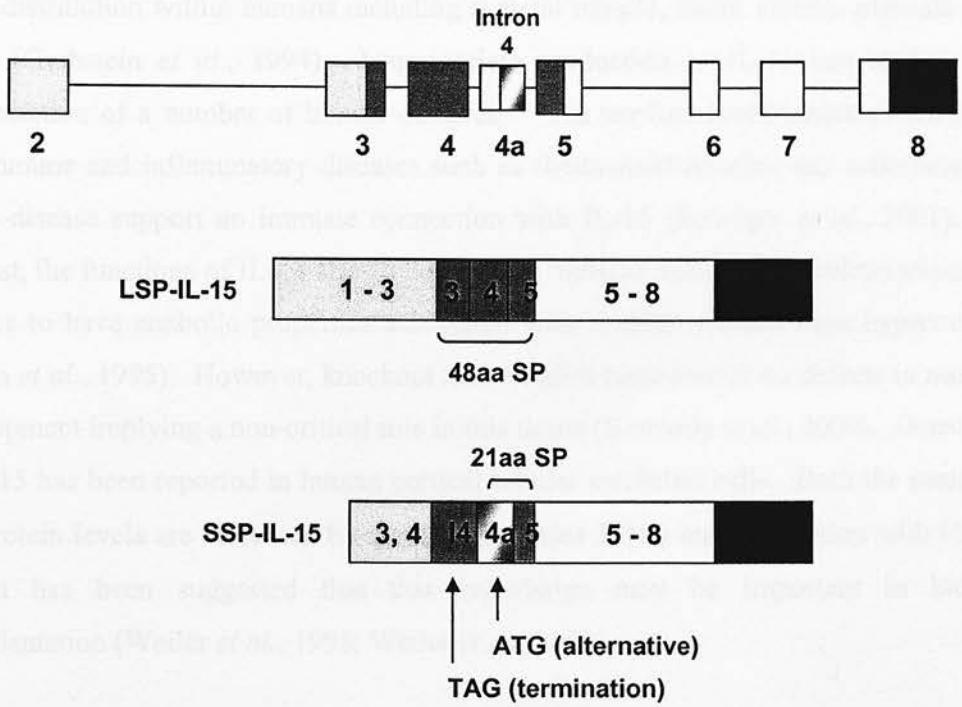


Figure 1.5

The generation of the two isoforms of IL-15, SSP and LSP, from the human IL-15 gene locus. Diagram recreated from Waldmann *et al* 2001 (Waldmann *et al.*, 2001).

1.4.3.1 Systemic IL-15

Human IL-15 has 97% sequence homology compared with simian IL-15 and 73% homology with murine IL-15 (Anderson *et al.*, 1995). This conservation in IL-15 between species suggests an important biological role. This is compounded by its wide tissue distribution within humans including skeletal muscle, heart, kidney, placenta and uterus (Grabstein *et al.*, 1994). Inappropriate production of IL-15 can lead to the manifestation of a number of human diseases. An implied involvement of IL-15 in autoimmune and inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease support an immune connection with IL-15 (Fehniger *et al.*, 2001). In contrast, the functions of IL-15 stretch beyond the immune system. In skeletal muscle it appears to have anabolic properties associated with skeletal muscle fiber hypertrophy (Quinn *et al.*, 1995). However, knockout mice studies have shown no defects in muscle development implying a non-critical role in this tissue (Kennedy *et al.*, 2000). Detection of IL-15 has been reported in human cortical tubular epithelial cells. Both the message and protein levels are increased by the Th-1 cytokine IFN-γ and by ligation with CD40 and it has been suggested that this knowledge may be important in kidney transplantation (Weiler *et al.*, 1998; Weiler *et al.*, 2001).

1.4.3.2 The Reproductive Relevance of IL-15

Although the actions of IL-2 and IL-15 are reported to be similar, their tissue distribution is distinct. The uterus and placenta were found to be negative for IL-2 (Jokhi *et al.*, 1994b; King *et al.*, 1995) and so further investigation into other cytokines that could stimulate uNK cells was undertaken. The novel cytokine, IL-15 was discovered via detection of both the mRNA (Okada *et al.*, 2000b; Verma *et al.*, 2000) and protein (Kitaya *et al.*, 2000). Gene profiling has demonstrated an upregulation of IL-15 during the human "implantation window" compared with the late proliferative phase. The IL-15 precursor, IL-15 and the α chain of the IL-15 receptor are all increased by at least three-fold (Kao *et al.*, 2002) and this supports the role of IL-15 in

the process of implantation. Other studies have also confirmed higher levels of IL-15 mRNA expression in the secretory phase versus the proliferative phase with highest expression levels in the mid-late secretory phase (Kitaya *et al.*, 2000; Okada *et al.*, 2000b; Verma *et al.*, 2000; Chaouat *et al.*, 2002). Immunohistochemistry localised IL-15 to the glandular and luminal epithelium during the proliferative phase and in the mid-late secretory phase to the perivascular stromal cells (Kitaya *et al.*, 2000). Studies on stromal cells grown *in vitro* imply that production of this cytokine is partly under hormonal control (Okada *et al.*, 2000a). However, the presence of IL-15 is not limited to the menstrual cycle but is also abundant in first trimester decidua (Kitaya *et al.*, 2000; Okada *et al.*, 2000b).

The human placenta is an important source of IL-15. Studies utilising a trophoblast invasion model, JEG-3, discovered IL-15 has the ability to enhance trophoblast invasion and migration *in vitro* (Zygmunt *et al.*, 1998) and it is postulated to be involved in this process *in vivo*. It is apparent that the ratio of IL-13: IL-15 is important in controlling this process *in vivo* since it has been discovered that women experiencing recurrent spontaneous abortion (RSA) expressed raised levels of IL-13 and IL-15 in endometrial stromal and epithelial cells with the ratio being in favour of IL-13 (Chegini *et al.*, 2002). In general terms it is accepted that a Th-2 response is beneficial to pregnancy (Wegmann *et al.*, 1993), however, the rise in IL-13 expression over that of IL-15 in RSA endometrium implicates complexity to this classification (Chegini *et al.*, 2002). The general hypothesis that Th-1 cytokines are detrimental and Th-2 cytokines are beneficial to pregnancy is probably an over-simplification (Chaouat *et al.*, 2002). This is of particular importance given the role of IFN-γ in the establishment of normal pregnancy in the mouse (Ashkar *et al.*, 1999; Ashkar *et al.*, 2000).

1.4.3.3 Studies on IL-15 Null Mice

Knockout mice for IL-15 (-/-) have no NK cells present and this suggests a role for IL-15 in the development and maturation of these cells (Kennedy *et al.*, 2000). Studies

specifically within the uterus have shown with knock-out studies in mice that mutant mice lacking uNK cells display irregular oestrous, deficient decidual formation and thickened blood vessel walls (Miyazaki *et al.*, 2002). In addition to these defects, a reduced placental size occurs in uNK cell deficient mice (Greenwood *et al.*, 2000). This supports the theory that these cells are involved in transformation of uterine vasculature (King *et al.*, 1990). In humans, inadequate artery transformation can result in pre-eclampsia, intra-uterine growth retardation (IUGR) and still birth and with the case of IUGR this programming *in utero* can be associated with cardiovascular diseases in adult life (Barker, 1997a; Barker, 1997b).

1.5 Hypothesis and Aims

Previous studies have identified various factors that are involved in the decidualisation of endometrial stroma, a process that is essential to a successful pregnancy in humans. The accumulation of uNK cells across the secretory phase coincides with this decidual transformation of the stromal compartment. The regulation of uNK cells and their specific functions in the human uterus are not fully understood although it is likely they are closely linked to the ESC that is under progesterone control. From the results of previous research discussed in section 1.2.2.1, a hypothesis of these speculative actions has been summarised in figure 1.6.

Paracrine interactions between uNK cells and ESCs via cytokines are likely to be essential to the respective development and functions of both cell types. Specific products of uNK cells, including IFN-γ and IL-10, may be modulating the function of ESCs in addition to maintenance of inflammatory and immune homeostasis. At this point in time the ESC cell will be transforming via decidualisation and products of the uNK cells may be modulating this conversion reaction. Reciprocally, cytokine production by ESCs will provide communication with uNKs and possibly amend their cytokine production. Feedback between these cell types will be essential to fine-tuning the control of these actions and thus establish an environment correctly prepared for pregnancy.

Prostaglandins are considered important to many of the key reproductive events in the human uterus although their exact effects are not yet clearly defined. Their actions are controlled at multiple levels within cells by the activity of various enzymes and this represents a complex cascade of events. PGE_2 is involved in decidualisation of the endometrial stroma, a process in humans essential to successful implantation. Expansion in our knowledge of the changes occurring to the PGE_2 pathway will provide a greater understanding of the mechanisms controlling the decidual transformation. This process is likely to be tightly regulated with modifications at a number of control points

along the cascade. The transport of PGE_2 across the cell membrane is a further control mechanism post-translationally and will be relevant to transport out of and into cells. In addition to actions on ESCs, PGE_2 can act to modify the actions of IL-15 on the cell surface of NK cells at the receptor level. PGE_2 may also be regulating the production of cytokines by uNK cells via actions on the IL-15 receptor or directly on its own receptors at the surface of the cell.

The Main Aims of this Research Project:

1. Further investigate decidualisation of human ESCs *in vitro* and explore the functional link between IL-15 and decidualisation.

2. Assess the changes in the prostaglandin cascade during decidualisation of ESCs.

3. Assess the potential factors that are involved in the regulation of IL-15 mRNA expression and protein secretion in ESCs *in vitro* and in the human endometrium and decidua.

4. Investigate the effects of PGE_2 and IL-15 on uNK cells *in vitro.*

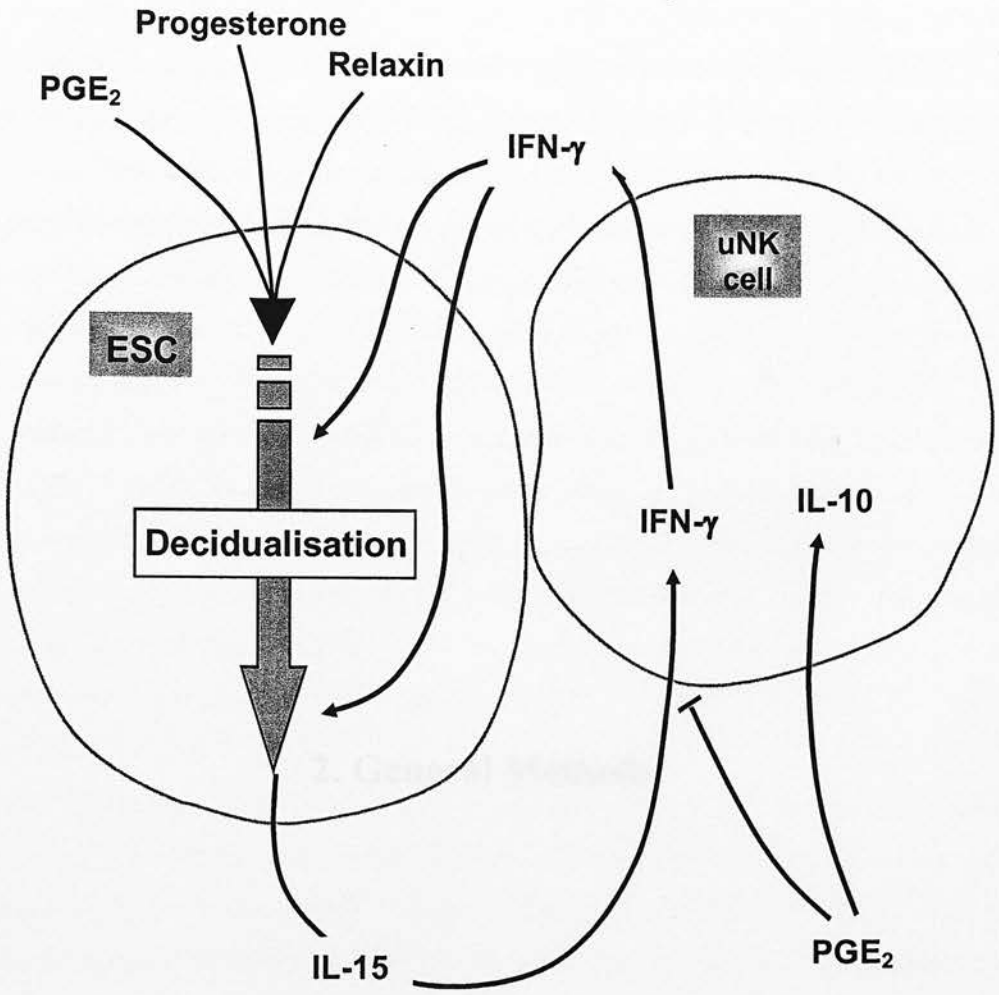


Figure 1.6

The hypothesised interactions between uNK cells and ESCs in the human endometrium/decidua during the secretory phase and in early pregnancy implicating the indirect action of progesterone on uNK cell function via the ESC. Studies on blood NK cells demonstrated that it is the $CD56^{bright}$ NK cells that may be the major producers of cytokines (Cooper *et al.*, 2001a). uNK cells may therefore represent an important uterine cell able to be stimulated to produce a variety of inter-cellular mediators.

2. General Methods

2.1 Collection, Processing of Human Uterine Tissue and Ethics

Human endometrial specimens were collected from women of reproductive age (n = 77) who were undergoing gynaecological procedures for benign indications. All women had regular menstrual cycles with a length of between 25-35 days and had not received exogenous hormones or used an intrauterine contraceptive device in the 3 months prior to surgery. For all endometrial biopsies analysed, the stage of the menstrual cycle was consistent with the patient's reported last menstrual period and histological dating using the criteria of Noyes *et al* (Noyes *et al.*, 1950). Samples were classified as menstrual (M), proliferative (P), and early (ES), mid (MS) and late secretory (LS) phases for the purposes of Taqman Q RT-PCR. Any cases with severe uterine pathology, for example, polyps or large fibroids, were excluded. All subjects had a serum sample collected at the time of surgery for the determination of circulating oestradiol and progesterone levels by RIA. All samples were consistent with the designated cycle stage based on morphological criteria and last menstrual period (see table 2.1) (Critchley *et al.*, 2002).

Decidual tissue was collected by two different methods for varying experimental procedures. For the purpose of the analysis of mRNA expression across the cycle decidual (n = 5) and trophoblast (n = 4) specimens were obtained from women who had undergone surgical termination by vacuum aspiration, during the first trimester of pregnancy. All patients had the termination performed under general anaesthesia. Prior to vacuum aspiration, decidual tissue distant from the implantation site was removed by gentle curettage of the uterine lining. Presence and absence of decidual parietalis and trophoblast respectively was confirmed by examination of haematoxylin and eosin stained tissue sections. Tissue sections were also stained with cytokeratin to confirm the presence or otherwise of trophoblast cells within the decidual tissue (Critchley *et al.*, 1996). The endometrial tissue was collected in sterile Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, Poole, Dorset, UK). The decidual tissue samples were processed for messenger RNA (mRNA) extraction alone. The endometrial samples were divided and processed as follows: (i) homogenisation in Tri Reagent® (a

monophase solution containing phenol and guanidine thiocynate) to allow mRNA isolation from DNA and protein; (ii) fixation in 10% neutral buffered formalin (NBF) overnight at 4°C followed by storage in 70% ethanol preceding wax embedding; and (iii) separation of endometrial stromal cells (ESCs) by sedimentation (see section 2.2). A second method of decidua collection was utilised in order to collect tissue for the uterine Natural Killer cell separation procedure. Insufficient amounts of tissue could be collected via the former method. In order to collect larger volumes of decidual tissue the total tissue yield from the suction curettage procedure (used to evacuate uterine contents) were collected. Decidual tissue (n = 15) was selected by macroscopic inspection from the products of the termination aspiration procedure (this process was performed by our trained, Research Sister). Thereafter decidual tissue was collected in sterile RPMI 1640 medium (Sigma, Poole, Dorset, UK) in preparation for the uterine Natural Killer cell separation procedure. A small section of tissue was placed in RNA Later (Ambion Europe Ltd., Cambridgeshire, UK) in order that mRNA could be later extracted. A second small section of tissue was placed into 10% NBF and following fixation for 24 hours samples were placed in 70% ethanol preceding wax embedding. Decidua was only collected from women with a pregnancy of gestation age between 7 and 10 weeks.

Informed patient consent was obtained prior to all tissue collection by a dedicated Research Nurse. Ethical local research approval had been previously granted by the Lothian Research Ethics Committee for all studies.

Cycle days/ weeks of gestation (decidua)	Cycle Stage	Number of tissue samples	Oestradiol (pmol/l) Median (range)	Progesterone (nmol/l) Median (range)
1 - 4	Menstrual	9	136 (57 – 306)	3.4 (1.6 – 4.4)
5 - 7	Early-proliferative	1	669	1.5
8 - 10	Mid-proliferative	19	570 (193 – 1235)	1.8 (1.3 – 15.5)
11 – 13	Late-proliferative	7	599 (211 – 1010)	4.4 (2.6 – 10.1)
14 – 18	Ovulatory/ Early-secretory	16	425 (264 – 1323)	15.4 (1.5 – 63.3)
19 – 23	Mid-secretory	9	368 (120 - 650)	33.7 (8.4 – 71.3)
24 – 28	Late-secretory	7	398 (189 – 477)	8.9 (1.1 – 28.1)
7 - 10	First trimester decidua	20	NA	NA

Table 2.1

Details of the endometrial and decidual biopsies studied in this research project. The median and range of serum oestradiol and progesterone concentrations are included.

2.2 Endometrial Cell Culture

Endometrial specimens (n = 28) were separated into epithelial and stromal cell populations by a sedimentation procedure. Specimens were initially washed in phosphate buffered saline and sliced into 1-2mm pieces using scalpel blades. These were re-suspended in 2ml PBS and digested in collagenase (Sigma) for 80 minutes at 37°C. An 18 gauge needle was used to facilitate tissue breakdown. The cells were then pelleted by centrifugation and re-suspended in 12ml RPMI 1640 and left for 5 minutes to settle. The top 10ml were removed as the population of endometrial stromal cells (ESCs). A Fluorescence Activated Cell Sorter (FACS) had been used to verify the purity of cells separated by this technique (see section 2.3). The ESCs were further cultured in RPMI 1640 medium (Sigma) supplemented with 10% foetal calf serum (FCS) (Mycoplex; PAA, Teddington, UK), penicillin (50 μg/ml; Sigma), streptomycin (50 μg/ml; Sigma) and gentamycin (5 μg/ml; Sigma). The endometrial specimens used in the cell culture experiments were predominantly from the proliferative phase. However, due to a shortage of specimens it was necessary to use some from the early secretory phases also. These specimens will have been exposed to progesterone *in vivo* although those from the proliferative phase had not. It was therefore necessary to grow all of the cultures in the prescence of progesterone prior to being used in the experiments so that all cultures had been exposed to progesterone. Cells were cultured in $75cm^3$ culture flasks (Corning Incorporated, Corning, NY) for a minimum period of 5 days and allowed to reach confluence in the presence of oestradiol (10^{-7} M), Medroxyprogesterone acetate (MPA) (10^{-6} M) and basic fibroblast growth factor (bFGF) (5ng/ml). These supplements also provide optimum conditions for ESC growth (Irwin *et al.*, 1991).

2.3 Fibroblast Cell Purity

Cultures of ESCs were grown for two weeks before the purity of fibroblasts in the cultures (n = 6) was determined by a Fluorescence Activated Cell Sorter (FACS)

(Beckmann Coulter). This was achieved by using a human fibroblast antigen Ab-1 (Oncogene). About 1 million ESCs were taken from an ESC culture following trypsinisation, re-suspended in 200µl Cell Separation Media (CSM) (PBS 0.5% bovine serum albumin; 2mM EDTA) and split between three 1.5ml Eppendorf tubes. Into one tube 2µl of the fibroblast antibody was added and mixed and the tubes were left on ice for 30 minutes. The tubes were spun at 4000rpm for 1 minute to pellet the cells, which were re-suspended in 1ml CSM. This wash was repeated twice more. To two tubes 100µl CSM and 10µl anti-mouse IgG whole molecule with FITC conjugated (Sigma) was added and cells re-suspended. The remaining tube is used as a "blank". The tube exposed to only the anti-mouse IgG whole molecule with FITC conjugated is the negative isotype-matched control. The tubes were wrapped in foil to prevent light exposure and kept on ice for 30 minutes. Tubes were washed as before and re-suspended in 500µl FACs buffer (PBS 0.1% Azide; 1% FCS). Before passing the cells through the FACS machine the cell suspensions were passed though cell strainers (Becton Dickinson Labware Europe, France) to ensure a single cell suspension. See figure 2.1 for an example of the FACS reports.

2.3.1 Fluorescence Activated Cell Sorter (FACS)

A Coulter ® EPICS® XL™ Flow Cytometer was the FACs machine used to measure the purity of the cells. This machine allows the purity of a specific cell type within a mixed population of cells to be measured. The cells are passed through an aperture as single cells. The laser beam detects cells and registers them as an event. The laser beam is able to determine two properties of each cell: the granularity and cell size. The granularity is read by the amount of side scatter of the laser beam and the cell size determined by the degree of forward scatter. The third measurement made by the laser beam is fluorescence, in this case fluorescein. The three measurements combined illustrate the distinct populations of cells that are present and the percentage of fluorescent gives the percentage of fibroblast-positive cells in the sample.

Figure 2.1

A representative example of the FACS recordings for the fibroblast purity of ESCs after 2 weeks in culture. The isotype-matched negative control (**A**) and the sample incubated with the fibroblast antibody (**B**). On graph (i) the y axis is the log of the forward scatter and the x axis is the log of the side scatter. The total number of counts is illustrated on this graph and the region within the red line represents the gated area of cells analysed. The remainding points that are outside of this region are likely to be cell debris or red blood cells and are therefore excluded from analysis. On graph (ii) the y axis is the total cell count and the x axis is the FITC value – the total level of fluorecence detected on the cells counted within the gated region in graph (i). The region represented by the green line indicates the number of cells that are positive for FITC. This is minimal in figure A (ii) (the negative control) whereas in figure B (ii) the majority of the cells are positive for FITC.

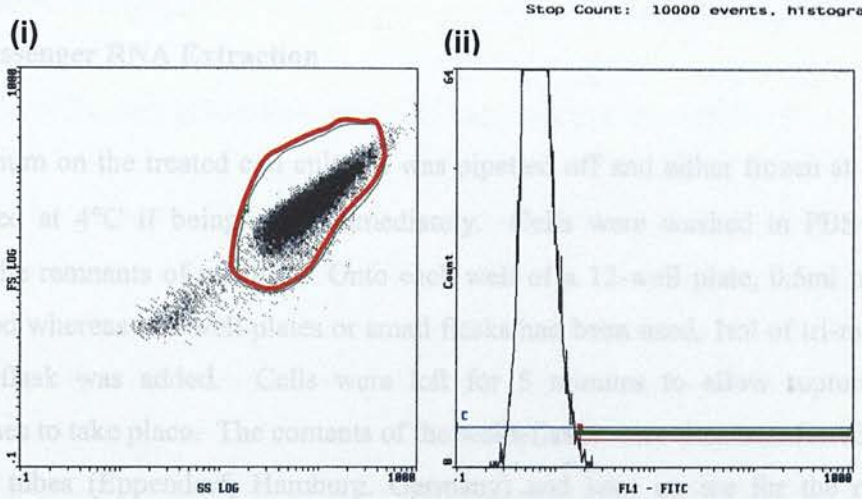
A **MRC EDINBURGH**

COULTER(R) EPICS(R) Acquisition Flow Cytometry Report

14May02 11:04:03
fibro 15.3.02
Z0012760
Endo150 neg 14.5.02 fibrc
31 seconds. 10817 events
Stop Count: 10000 events, histogram 2

OP ID: SJD

Initial cytosett. from prot. fibro 15.3.02



Stats: Normalized, Listgating: Disabled

Color equations

Hist	Region	ID	%	Count	Mn X	Mn Y	PkPosX	PkPosY	PkCnt	FPCVX	FPCVY
1	A	A	92.4	10000	77.2	38.5	71.5	40.2	65	61.62	56.00

Hist	Region	ID	%	Count	Mn X	Md X	PkPosX	PkCnt	HPCV	Min	Max
2	B	B	1.34	134	2.12	1.80	1.62	8	0.69	1.61	1024
	C	C	100	10000	0.673	0.648	0.602	150	18.25	0.102	996.7

B **MRC EDINBURGH**

COULTER(R) EPICS(R) Acquisition Flow Cytometry Report

14May02 11:05:38
fibro 15.3.02
Z0012761
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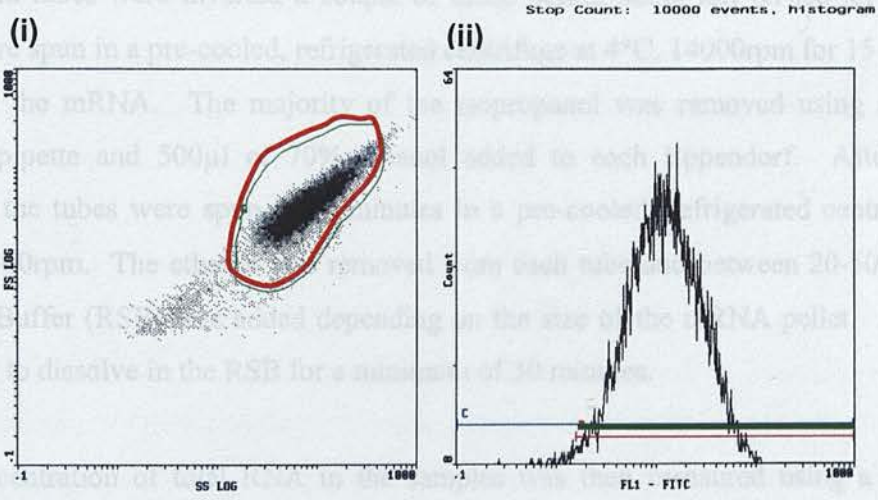
Stop Count: 10000 events, histogram 2

OP ID: SJD

Initial cytosett. from prot. fibro 15.3.02



Stats: Normalized, Listgating: Disabled

Color equations

Hist	Region	ID	%	Count	Mn X	Mn Y	PkPosX	PkPosY	PkCnt	FPCVX	FPCVY
1	A	A	93.7	10000	80.7	39.7	71.5	40.2	62	63.05	55.95

Hist	Region	ID	%	Count	Mn X	Md X	PkPosX	PkCnt	HPCV	Min	Max
2	B	B	98.9	9886	12.5	12.5	10.9	57	6.44	1.61	1024
	C	C	100	9999	12.2	12.4	10.9	57	6.44	0.102	996.7

2.4 Messenger RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

2.4.1 Messenger RNA Extraction

The medium on the treated cell cultures was pipetted off and either frozen at –20°C or maintained at 4°C if being used immediately. Cells were washed in PBS twice to remove the remnants of medium. Onto each well of a 12-well plate, 0.5ml tri-reagent was added whereas if 6-well plates or small flasks had been used, 1ml of tri-reagent per well or flask was added. Cells were left for 5 minutes to allow rupture of cell membranes to take place. The contents of the wells/flasks were then transferred to phase lock gel tubes (Eppendorf, Hamburg, Germany) and kept on ice for the remaining extraction. Chloroform was added at 200µl/ml and the tubes shaken vigorously for several seconds. They were spun in a pre-cooled, refrigerated centrifuge at 4°C, 14000rpm for 20 minutes to remove the organic phase containing protein and DNA that become trapped beneath the gel layer. The top aqueous phase containing the mRNA was poured into labelled 1.5ml eppendorfs. Into each tube 500µl of isopropanol was added and tubes were inverted a couple of times before being left on ice for 1 hour. They were spun in a pre-cooled, refrigerated centrifuge at 4°C, 14000rpm for 15 minutes to pellet the mRNA. The majority of the isopropanol was removed using a sterile Pasteur pipette and 500µl of 70% ethanol added to each Eppendorf. After being inverted the tubes were spun for 5 minutes in a pre-cooled, refrigerated centrifuge at 4°C, 14000rpm. The ethanol was removed from each tube and between 20-50µl RNA Storage Buffer (RSB) was added depending on the size of the mRNA pellet. Samples were left to dissolve in the RSB for a minimum of 30 minutes.

The concentration of total RNA in the samples was then measured using a Biotech photometer (WPA, Cambridge, UK). Each sample was vortexed and 2.5µl removed, which in turn was diluted in 2ml distilled water. All tubes were thoroughly mixed before measuring the wavelength at both 260nM and 280nM on the photometer. A

blank used to zero the machine consisted of 2.5µl RSB diluted in 2ml of distilled water. All samples were then diluted in Diethylpyrocarbonate-treated water (Depc water) to a concentration of 100ng/µl.

2.4.2 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Messenger RNA samples (at 100ng/µl) were reversed transcribed using a multiscribe reverse transcriptase (1.25 IU/µl), random hexamers (2.5 µmol/l), $MgCl_2$ (5.5 mmol/l), dNTPs (1mmol/l) and an RNAase inhibitor (0.4 IU/µl) was added (PE Biosystems, Warrington, UK). All reactions consisted of 16µl of reverse transcription mix per tube and 4µl of mRNA. Once the contents of the tube were mixed, 50µl of mineral oil was added to prevent evaporation of the reaction mix during the reverse transcription process. Samples were incubated for 20 minutes at 25°C, 60 minutes at 42°C and then at 95°C for 5 minutes (Omnigene PCR machine, UK). The resulting cDNA was then diluted 2.5x with TE buffer (10mmol/l Tris pH8.0 and 1mmol/l EDTA in DEPC water) and were stored at 4°C.

The precision of the reverse transcription (RT) reaction was calculated by a colleague, Elena Faccenda (laboratory research support). An mRNA sample was taken and 8 RT reactions set up in 8 separate tubes. These were compared in a single PCR run on one primer and probe set. The precision was found to be 3.65%.

2.5 Quantitative Real-time-PCR (Q RT-PCR)

This procedure allows the quantitative detection and measurement of a specific sequence of complementary DNA (cDNA). Primers and probes were designed individually for each DNA target sequence using the Primer Express computer program. The probe has a reporter dye and quencher dye attached to the 5' and 3' ends respectively. Whilst intact, the close proximity of the reporter to the quencher suppresses fluorescence of the reporter. If the target sequence is present the probe anneals to the cDNA in between the

blank used to zero the machine consisted of 2.5µl RSB diluted in 2ml of distilled water. All samples were then diluted in Diethylpyrocarbonate-treated water (Depc water) to a concentration of 100ng/µl.

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This procedure allows the quantitative detection and measurement of a specific sequence of complementary DNA (cDNA). Primers and probes were designed individually for each DNA target sequence using the Primer Express computer program. The probe has a reporter dye and quencher dye attached to the 5' and 3' ends respectively. Whilst intact, the close proximity of the reporter to the quencher suppresses fluorescence of the reporter. If the target sequence is present the probe anneals to the cDNA in between the

forward and reverse primer sites and this is referred to as the polymerisation step. The sequence of either strand must be complementary to the probe for it to attach. The Taq polymerase has nuclease activity in the 5' to 3' direction and cleaves the probe in between the reporter and quencher. This can only take place if the probe has hybridised to the target sequence of cDNA. Cleavage of the probe allows separation of the reporter dye from the quencher dye with the result being an increase in fluorescence. This increase in fluorescence allows the accumulation of PCR products to be measured directly.

The FAM Ct is the cycle number when the fluorescent signal crosses an arbitrary threshold value. The 18-S is a measure of the mRNA content of the sample and is used as an internal control for mRNA variation between samples. The ΔCt is the difference between the FAM Ct and the 18-S reading and enables the amplified signal to be normalised against the total mRNA content. The mean ΔCt between the duplicates on the PCR plate was calculated. This was then used to calculate the ΔΔCt which is the difference between the ΔCt of a treated sample relative to the control in that experiment. Within each experiment the ΔCt was related to its own control. The $^{2^-}$ΔΔCt is then calculated which shows the fold increase or decrease in mRNA expression of the samples in relation to their control with each control always having a $^{2^-}$ΔΔCt of 1.

All primer and probes sets used on the ABI Prism 7700 PCR machine were purchased from Biosource (Belgium). The sequences for these are listed in table 2.2. Primers were diluted to 250μM and probes to 50μM in TE buffer (10mM Tris; 1mM EDTA in Depc H_2O). A PCR master mix was made up using the Stratagene Brilliant Quantitative PCR Core Reagent kit (Amsterdam, Netherlands) (7.2mM $MgCl_2$; 1.6mM Stratagene dNTP mix; 1.6mM Boehringer dNTP mix; 0.05U/μl Taq Polymerase; 2x PCR Buffer and 0.06% Reference dye diluted in Depc H_2O). Sample to be tested were ran in duplicate wells on the PCR plate (Applied Biosystems, UK). For each sample, a tube containing 45μl PCR Master Mix and 5μl cDNA was mixed and 23μl pipetted into two separate

wells on the PCR plate. Two wells containing 5µl of Depc H_2O in place of cDNA were added to each run to serve as a negative control.

The primer and probe sets used were validated with respect to the linearity of the response. Graphs of the log of total RNA in ng were plotted against the mean ΔCt value of three replicates. The regression line of the graph (y) needed to be < 0.1 in order that the primers and probe could be validated (Figure 2.2). The intra-assay variation was calculated as a precision value (relative standard deviation) calculated from the mean of six replicates of identical cDNA ran on the PCR machine in the same run. This value is expressed as a percentage. (See table 2.3).

Table 2.2

Details of the primer and probe sets used in Q RT-PCR in this study. The accession number and sequences for the primers and probe are detailed.

Amplicon	Accession Number	Forward Primer	Reverse Primer	Probe
β-actin	X00351	TCACCCACACTGTGCCCATCTACGA	CAGCGGAACCGCTCATTGCCAATGG	ATGCCCCCCCCATGCCATCCTGCGT
CD40L	Z15017	GTGCTTCGGTGTTTGTCAATGT	CAGCCTGCAAGGTGACACTGT	ACTGATCCAAGCCAAGTGAGCCATGG
COX-1	S78220	TGTTCGGTGTCCAGTTCCAATA	ACCTTGAAGGAGTCAGGCATGAG	CGCAACCGCATTGCCATGGAGT
COX-2	M90100	GTGTTGACATCCAGATCACATTTGA	GAGAAGGCTTCCCAGCTTTTGTA	TGACAGTCCACCAACTTACAATGCTGACTATGG
EP2 Receptor	U19487	GACCGCTTACCTGCAGCTGTAC	TGAAGTTGCAGGCGAGCA	CCACCCTGCTGCTGCTTCTCATTGTCT
EP4 Receptor	D28472	ACGCCGCCTACTCCTACATG	AGAGGACGGTGGCGAGAAT	ACGCGGGCTTCAGCTCCTTCCT
IFN-γ	X13274	CCAACGCAAAGCAATACATGA	TTTTCGCTTCCCTGTTTTAGCT	CTCATCCAAGTGATGGCTGAACTGTCGC
IGFBP-1	M59316	CACAGGAGACATCAGGAGAAGAAA	ACACTGTCTGCTGTGATAAAATCCAT	TTCCAAATTTTACCTGCCAAACTGCAACAA

Amplicon	Accession Number	Forward Primer	Reverse Primer	Probe
IL-15	U14407	GTATTGTA GGAGGCAT CGTGGAT	TAGCACTG GATGGAAA TACTTCTCA	ATGGCTGC TGGAAACC CCTTGCC
PDE3a	M91667	GCAGCTGT GGCAGACC ATATT	CCGAGTGG CTACCCCA CTT	AATCCTGC TGATGAGC CCCTGGAG A
PDE4b	L20971	CCTTCAGTA GCACCGGA ATCA	CAAACAAA CACACAGG CATGTAGTT	AGCCTGCA GCCGCTCC AGCC
PGTP	U70867	CTGTGGAG ACAATGGA ATCGAGTA	CAAATAGA TCAGTTGC TTGGAGGTT	CTCCCCTT GCCATGC CGGC
Progesterone Receptor (genomic)	M15716	CAGTGGGC GTTCCAAA TGA	TGGTGGAAT CAACTGTAT GTCTTGA	AGCCAAGC CCTAAGCC AGAGATTC ACTTT
Prolactin	NM 000948	GCCCCGGA GGCTATCC TA	TCAGCTCCA TGCCCTCTA GAA	CCAAAGCT GTAGAGAT TCAGGAGC AAACCA

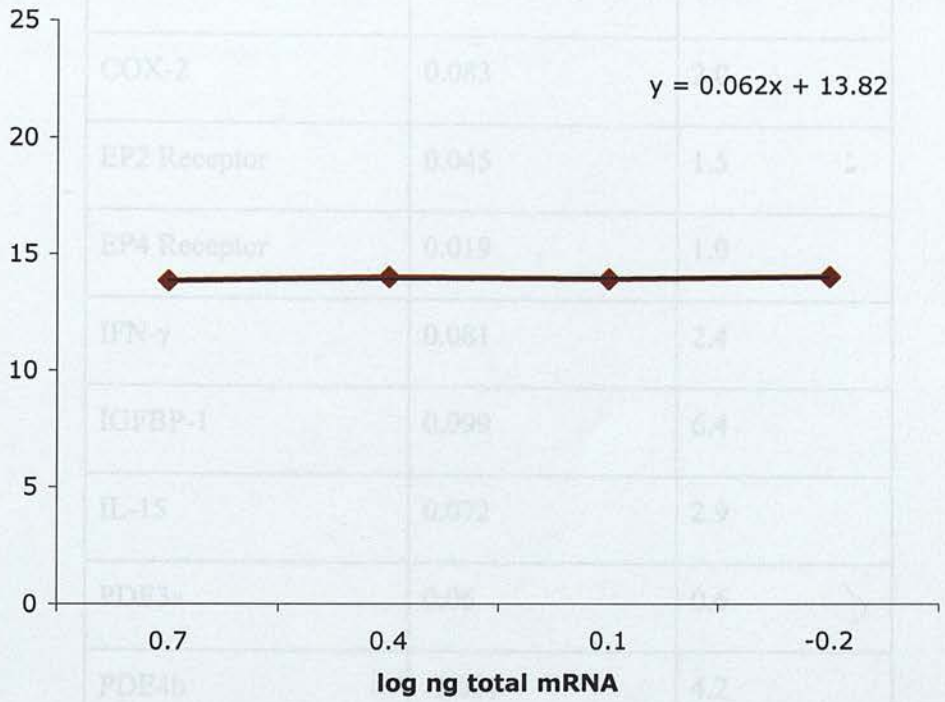


Figure 2.2

An example of a linearity plot for the primer and probes used for Q RT-PCR. This figure is the plot for the prolactin primer and probes. The y value for this particular plot is 0.062 and is an indication of the linearity.

Primer and Probe set	Linearity (y value)	Precision (%)
CD40L	0.057	7.3
COX-1	0.053	1.6
COX-2	0.083	2.0
EP2 Receptor	0.045	1.5
EP4 Receptor	0.019	1.0
IFN-γ	0.081	2.4
IGFBP-1	0.099	6.4
IL-15	0.072	2.9
PDE3a	0.06	0.6
PDE4b	-0.001	4.2
PGTP	0.073	1.3
Progesterone Receptor (nuclear)	0.004	0.8
Prolactin	0.062	2.4

Table 2.3

Validation and precision values for the primer and probe sets used in the present study.

To ensure that no genomic DNA contamination was present in the mRNA samples, the β-actin level was measured in the controls from each experiment and in all the RNA samples used for analysis across the menstrual cycle. The samples were not processed for the reverse transcription stage and were measured directly by Q RT-PCR in place of adding cDNA. An arbitrary level of 27, which was 3 standard deviations away from the mean of all samples, was set and any endometrial samples falling below this level were excluded from analysis. One sample was excluded for this reason. Figure 2.3 represents the β-actin measurements for the samples included in this study.

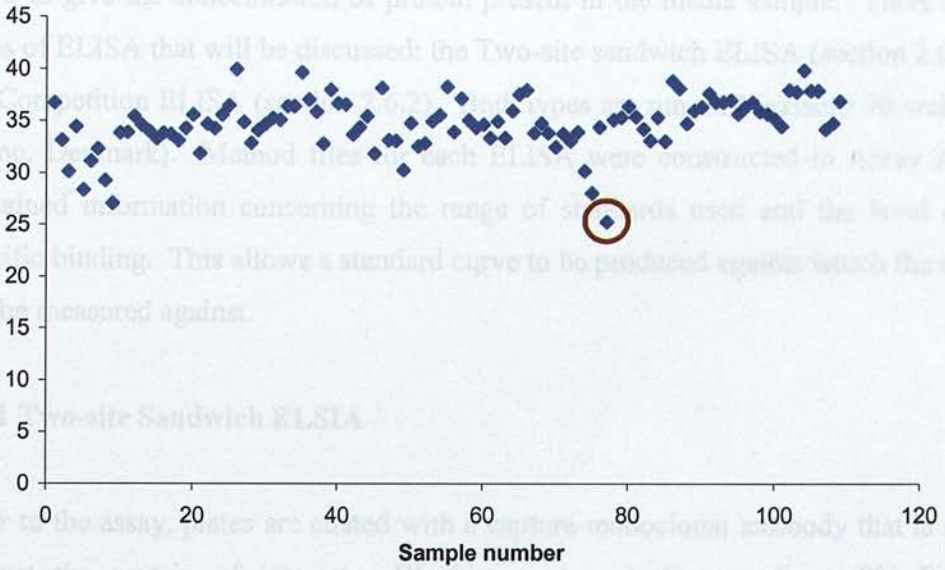


Figure 2.3

The β-actin levels for the experimental control mRNA samples and for the samples used in across the menstrual cycle analyses. The cycle number represents the fluorescence threshold level for all of the samples. Any samples with a value below 27 were excluded from the study. Therefore, the sample circled in red was omitted.

2.6 Enzyme-linked Immunoabsorbant Assay (ELISA)

An enzyme-linked immunoabsorbant assay, an ELISA, can be used to detect and measure various proteins released by cells during *in vitro* culture. The media in which the cells have been grown and treated was collected and stored at -20°C. The ELISA detects the amount of a particular protein present and measures this against a standard curve to give the concentration of protein present in the media sample. There are two types of ELISA that will be discussed: the Two-site sandwich ELISA (section 2.6.1) and the Competition ELISA (section 2.6.2). Both types are run on Maxisorp 96-well plates (Nunc, Denmark). Method files for each ELISA were constructed in Assay Zap and contained information concerning the range of standards used and the level of non-specific binding. This allows a standard curve to be produced against which the samples can be measured against.

2.6.1 Two-site Sandwich ELSIA

Prior to the assay, plates are coated with a capture monoclonal antibody that is specific against the protein of interest. Blocking and protecting medium (2% Polyvinyl Pyrollidone; 5mg/ml BSA; 0.5ml/litre Preservatives (200mg/ml 5-bromo-5-nitro-1, 3-dione and 200mg/ml 2-methyl-4-isothiazolin-3-one in DMF/DMSO 1:2); 5mM EDTA; 50mM EDTA) is added to prevent non-specific binding and reduce background recordings across all samples. The samples are incubated with the standards, quality controls (of a set concentration in multiple replicates), non-specific binding wells and finally the media samples to be assayed. Any free, specific protein in the wells will spontaneously bind to the capture antibody on the base and sides of the well. Excess media is discarded and washed away before a biotinylated detection antibody (secondary) is added. This will bind to the protein already bound on the plate and this is detected with streptavidin peroxidase conjugate (SPC), diluted in assay buffer (see specific assays for details). After washing the plates once more to remove any unbound SPC, substrate is added. The peroxidase substrate consisted of 20ml sodium acetate

(pH 6; 100mM), 2ml 0.5% urea hydrogen peroxidase (pH 6) and 2ml tetramethyl benzidine (3mg/ml). Any bound peroxidase will metabolise the hydrogen peroxide present in the substrate and the tetra methyl benzidine will be converted into a coloured product. The reaction is stopped by addition of 2N sulphuric acid. The colour is then measured on a plate reader at 450nM. The details of this ELISA are summarised in figure 2.4.

Stage 1:

The capture antibody is bound to the base and sides of the wells.

Stage 2:

Any antigens in the media specific to the capture antibody bind.

Stage 3:

A biotin-labelled detection antibody is added and it binds to the bound antigen.

Stage 4:

Streptavidin peroxidase conjugate is added to the wells and this converts the substrate to a coloured product that can be measured.

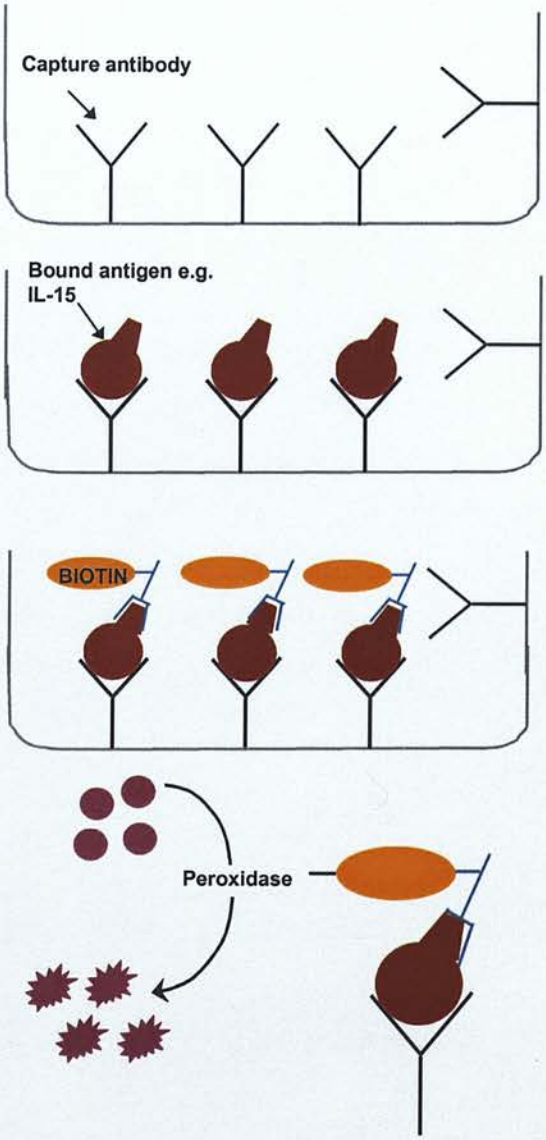


Figure 2.4

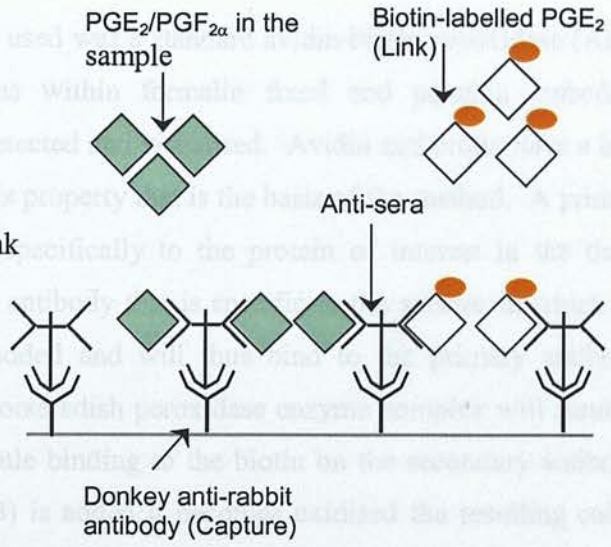
Description of the stages and principles of a two-site sandwich ELISA.

2.6.2 Competition ELISA ($PGF_{2\alpha}$ and PGE_2)

A Competition ELISA (see figure 2.5) works on a different basis to the Two-site sandwich ELISA. Initially plates are coated with a purified donkey anti-rabbit antibody (Sapu, Scotland, UK). Blocking and protecting medium is added to block any non-specific binding and reduce background. For the PGE_2 ELISA, a PGE_2 anti-sera (AS) raised in rabbit is added to the wells and this will bind to PGE_2. Since the coating antibody is anti-rabbit, this will also bind with the AS. Biotinylated labelled PGE_2 is added at the same time and this is referred to as the Link. Samples are added in this same step and this allows the PGE_2 protein in the sample to compete with the Link for binding sites on the AS. As in other ELISAs, a standard curve and quality control wells are added to the plate. The standard contains unlabelled PGE_2 that will compete for biding sites on the AS in the same way as for the unknown samples. The maximum binding is measured by addition of the Link and AS in duplicate wells and this is referred to as the B_0 value. The assay works on the principle that the greater the level of PGE_2 in the sample the greater the amount of displacement of the Link. This results in lower streptavidin binding and thus less colour product in the wells.

Stage 1:

The $PGE_2/PGF_{2\alpha}$ in the sample competes with the biotin-labelled link for anti-sera biding sites.



Stage 2:

The streptavidin peroxidase binds to the biotin on the bound link and converts the substrate into a coloured product that can be measured. The greater the level of $PGE_2/PGF_{2\alpha}$ in the sample, the lower the concentration of coloured product.

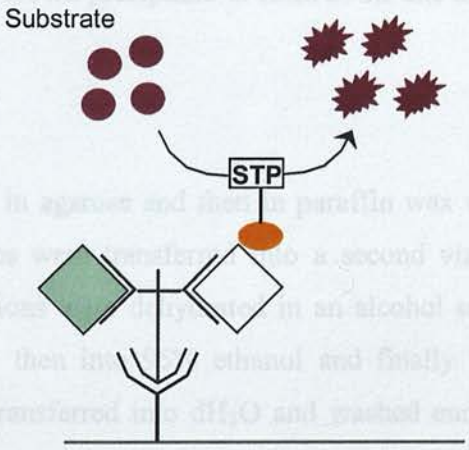


Figure 2.5

Description of the stages and the principles of a Competition ELISA.

2.7 Immunohistochemistry

The immunohistochemical method used was a standard avidin-biotin peroxidase (ABC) method allowing specific proteins within formalin fixed and paraffin embedded endometrial tissue sections to be detected and visualised. Avidin and biotin have a high affinity for one another and it is this property that is the basis of the method. A primary antibody is added and will bind specifically to the protein of interest in the tissue section. A biotinylated secondary antibody that is specific to the species in which the primary antibody was raised is added and will thus bind to the primary antibody. Addition of an avidin-biotin and horseradish peroxidase enzyme complex will result in any free sites on the avidin molecule binding to the biotin on the secondary antibody. When 3,3 diaminobenzidine (DAB) is added it becomes oxidised the resulting colour change of the chromagen causes an insoluble brown precipitate to form at the site of the protein of interest.

2.7.1 Preparation

Tissue sections and cultured cells embedded in agarose and then in paraffin wax were dewaxed in histoclear for 5 minutes. Slides were transferred into a second vial of histoclear for a further 5 minutes. The sections were dehydrated in an alcohol series starting in absolute ethanol for 20 seconds, then into 95% ethanol and finally 70% ethanol, each for 20 seconds. Slides were transferred into dH_2O and washed once in PBS.

2.7.2 Scoring of Immunohistochemistry

A semi-quantitative scoring system was used to measure the location and intensity immunostaining. The sections were scored blind and individually by two observers. The following system was employed: 0 = no immunoreactivity; 1 = faint immunoreactivity; 2 = moderate immunoreactivity; 3 = strong immunoreactivity. All of the slides analysed were included in the same immunohistochemical run and therefore a

fair comparison could be made between them. Initially all of the slides were examined briefly to assess the extremes in staining intensity. The staining intensity and area of staining across each slide was considered as a whole before deciding on the score value to give it. The two observers then came together and compared the scores they had asigned. If there were any discrepancies the slides were re-examined and a mutal decision made on the score that should be given.

2.8 Statistical Analysis

Significant difference of the Q RT-PCR and ELISA data was determined by analysis of variance (ANOVA) using the computer package Statview 3.0. Individual differences were assigned using Fisher's protected least square differences (PLSD) test.

The IL-15 immunohistochemistry results were analysed by Kruskal-Wallis analysis and Dunn's Multiple Comparisons Test used to assign significance (Instat 2.03). This approach was necessary since the data is non-continuous.

3. Features of Endometrial Decidualisation – *in vitro* studies

3.1 Introduction

The sequential development of the human endometrium is a prerequisite for successful implantation and continued pregnancy (Wilcox *et al.*, 1999; Lessey, 2000). In humans, the stromal compartment of the endometrium experiences a transformation across the mid- and late-secretory phases of the menstrual cycle referred to as decidualisation. This begins within the perivascular stromal cells and spreads throughout the stromal region contributing to the formation of decidua in pregnancy. The cells become larger and more rounded in appearance (figure 3.1) with intracellular structural rearrangements and increasing stromal oedema. The major *in vivo* trigger of these adaptations is considered to be progesterone acting upon oestrogen-primed cells (Milligan *et al.*, 1995; King, 2000). The differentiation of these cells runs parallel with the rise in progesterone levels across the secretory phase. Studies *in vitro* on primary cultures of human endometrial stromal cells (ESCs) have revealed that this process is complex implicating the involvement of other factors such as PGE_2 (Frank *et al.*, 1994), relaxin (Tabanelli *et al.*, 1992; Lane *et al.*, 1994) and cAMP (Tang *et al.*, 1993a; Yee *et al.*, 1993; Brar *et al.*, 1997) in addition to progesterone. More recently, Activin A, a member of the TGF-β superfamily, has been implicated as another inducer of decidualisation in ESC at least *in vitro* (Jones *et al.*, 2002) (Figure 3.1).

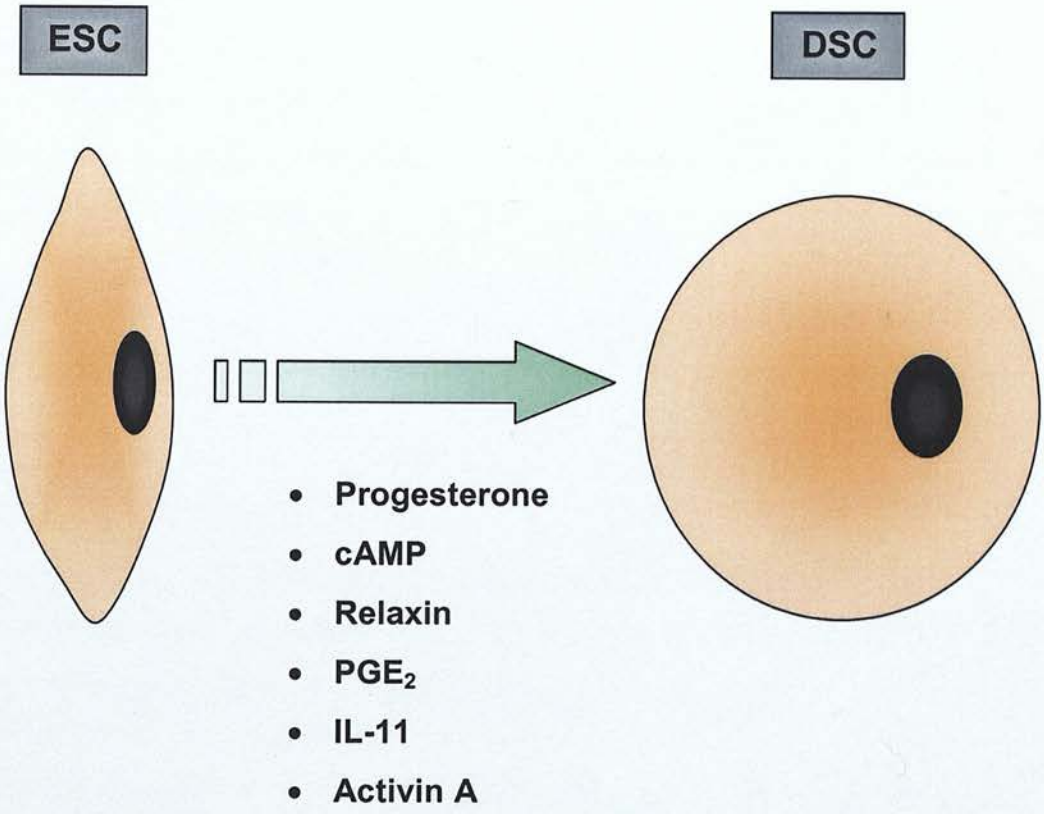


Figure 3.1

Diagrammatic illustration of the various factors thought to be involved in decidualisation of human ESCs to decidualised stromal cells (DSC), highlighting the multi-factorial nature of the process.

PGs are synthesised from the intermediate, PGH_2, (see sections 1.4.1 and 2.1 for details) by specific PG synthases (Smith *et al.*, 1996; Smith *et al.*, 1996; Murakami *et al.*, 2003). PGE_2 and PGE_2 synthase are located in glandular, stromal and epithelial cells in the functional layer of the human endometrium at all cycle stages with a reduction in stromal staining in the late secretory phase (Milne *et al.*, 2001). Prostaglandins may be important in the secretory phase and specifically during the implantation window since the EP2 receptor is up-regulated 4-fold compared with the late proliferative phase as shown by gene profiling (Kao *et al.*, 2002). There are four sub-types of the PGE_2 receptor, EP1-4 and activation of the EP2 and EP4 receptors (section 1.4.1) leads to stimulation of adenylate cyclase and the cAMP/protein kinase A pathway (Narumiya *et al.*, 1999). Relaxin is a phosphodiesterase inhibitor and therefore has the ability to maintain intracellular cAMP levels by inhibiting the action of phosphodiesterases, which breakdown cAMP. The principal source of relaxin in the female reproductive tract is the corpus luteum in the luteal phase and throughout pregnancy (Weiss *et al.*, 1978; Blankenship *et al.*, 1994; Bani, 1997; Sunder *et al.*, 2000). Relaxin is also expressed in the endometrium and decidua (Bryant-Greenwood *et al.*, 1993). Epithelial and stromal cells have been reported to display immuno-reactivity for relaxin with an increase in intensity across the secretory phase and throughout pregnancy.

It is not only the morphology of the stromal cells that transforms with decidualisation, but also their function. The stroma provides more extensive functions than a mere supportive structural role. It is believed that decidualised stromal cells are important in providing homeostasis during implantation and trophoblast invasion (Lockwood *et al.*, 1999). Progesterone receptor knock-out mice fail to exhibit decidualisation and this is associated with implantation failure (Rider, 2002). Trophoblast invasion in humans is significantly deeper in areas where the uterus is deficient in decidua caused by scar tissue. Imbalances in implantation and placentation can result in uterine rupture or the later development of pre-eclampsia (Loke *et al.*, 1997) highlighting the necessity for tight regulation of these events. The depth of trophoblast invasion in humans is under the influence of hCG, progesterone and oestradiol (Mitreski *et al.*, 2003) and in ectopic

pregnancies, the extent of trophoblast infiltration has also been shown to be influenced by hCG levels (Natale *et al.*, 2003). In humans, whether the process of decidualisation is irreversible is a matter for discussion and will be addressed in this chapter. Relaxin has previously been shown to acutely and permanently raise intracellular cAMP levels in cultured ESCs, resulting in prolactin secretion (Telgmann *et al.*, 1997).

Documented markers of the decidualisation process include prolactin (Maslar *et al.*, 1979; Tseng *et al.*, 1999) and IGFBP-1 (Bell *et al.*, 1991; Bryant-Greenwood *et al.*, 1993; Lee *et al.*, 1997) and these can be used as guidelines to observe and identify decidualisation *in vitro*. In humans, prolactin levels start to increase in early secretory phase endometrial stroma reaching maximal levels in decidua as shown by immunohistochemistry (Bryant-Greenwood *et al.*, 1993). This study also revealed a rise in IGFBP-1 immuno staining with a different timing of appearance to that of prolactin. IGFBP-1 was absent from proliferative and early secretory phase endometrium but began to appear in the mid secretory phase followed by a dramatic rise in the late secretory stroma (Figure 3.2). Other markers of decidualisation that have been identified in human and rat decidual stroma include vimentin, desmin, laminin and fibronectin (Glasser *et al.*, 1986; Glasser *et al.*, 1987; Van Muijen *et al.*, 1987) and these represent structural rearrangements within the cells.

The number of uNK cells present in the human endometrium increases across the secretory phase, particularly in the mid-secretory phase. This implies hormonal control over these cells (King *et al.*, 1989b) although they do not express the nuclear progesterone receptor (King *et al.*, 1996; Henderson *et al.*, 2003). Studies on uNK cells have demonstrated the ability of the cytokine IL-15 to stimulate proliferation of these cells *in vitro* (Verma *et al.*, 2000). A relationship between IL-15 and progesterone-induced decidualisation of human ESCs *in vitro* has been proposed (Okada *et al.*, 2000a). This provides a link between the functions of ESCs and uNK cells within the endometrium that may be important to successful embryo implantation and early pregnancy maintenance.

This chapter examines decidualisation in ESC cultures *in vitro*, studying the effects of treatment with a synthetic progestin, PGE_2 and 8-Bromo cAMP on the expression and release of prolactin and IGFBP-1. Using these data as an indication of decidualisation in these cultures a comparison between IL-15 mRNA expression and decidualisation *in vitro* has been made. Levels of the genomic progesterone receptor expression in these cells have been established. The effect of withdrawing decidualisation treatment on prolactin expression is also examined.

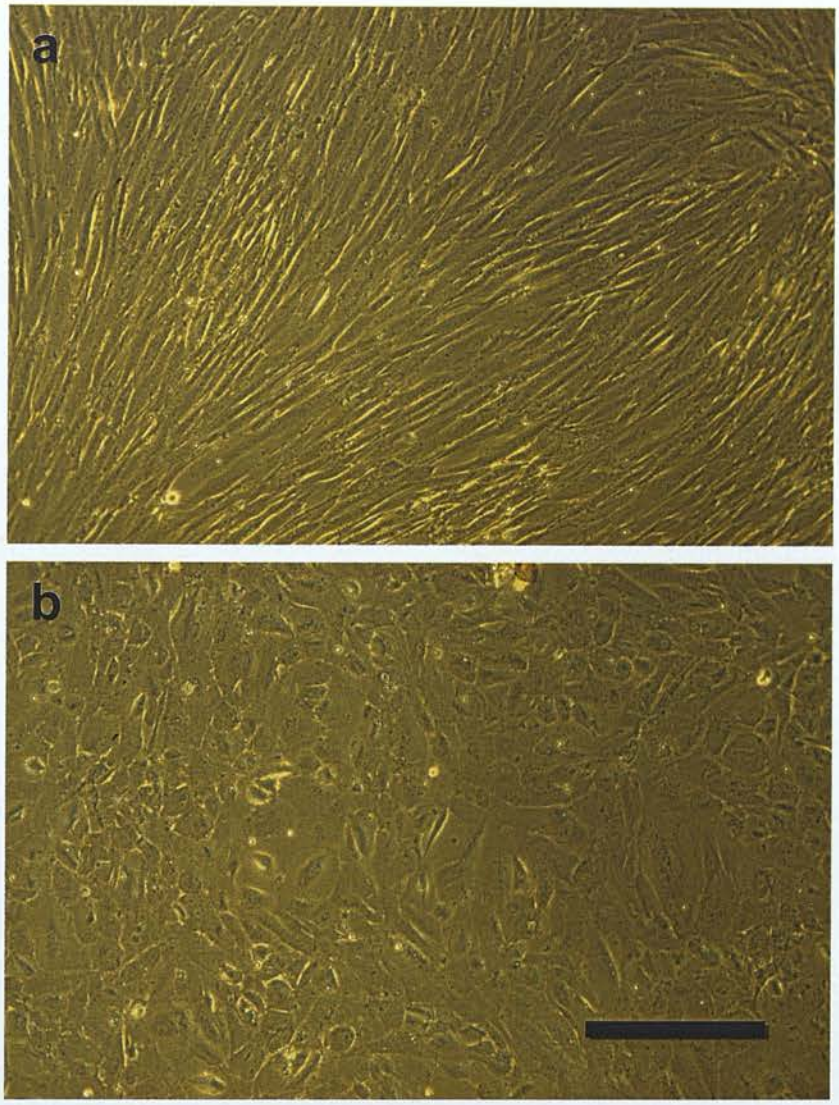


Figure 3.2

Photomicrographs of non-decidualised ESC (**a**) and decidualised ESC (**b**) primary cultures. The decidualised ESCs were treated for 6 days with a synthetic progestin, MPA, (10^{-6} M) plus 8-Bromo cAMP (250μM) and have become much rounder in appearance compared with the non-decidualised ESCs. Scale bar represents 300μm.

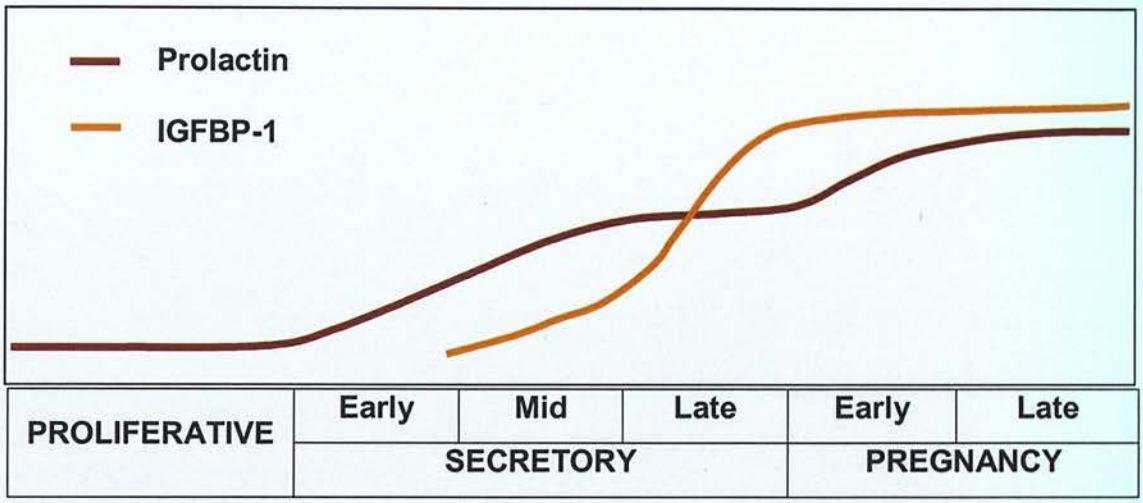


Figure 3.3

Schematic diagram of the variation in stromal immuno staining intensity of prolactin and IGFBP-1 across the human menstrual cycle and throughout pregnancy. Based on data from Bryant-Greenwood *et al* 1993 (Bryant-Greenwood *et al.*, 1993).

3.2 Materials and Methods

3.2.1 Human Uterine Tissue Collection

Proliferative (n = 5) and early secretory (n = 4) endometrial biopsies were collected and processed as described earlier (section 2.1). All samples were processed to separate the ESCs from the glands, as described in section 2.2. The cells were grown for a period of two weeks prior to commencing experiments in the presence of Medroxyprogesterone acetate (MPA) and bFGF.

3.2.2 *in vitro* Primary Cell Culture Studies – Decidualisation

ESCs were seeded in 12-well plates at a concentration of $1.4x10^5$ cells/ml and allowed to adhere overnight. The following treatment regimes were employed as detailed in tables 3.2 and 3.3. The experiments were designed to study *in vitro* decidualisation of ESCs. MPA is a synthetic progestin and is relatively stable. It was selected as a treatment to analyse the effects of a progestin alone on *in vitro* decidualisation. Both PGE_2 and synthetic cAMP analogues have previously been acknowledged as stimulators of decidualisation *in vitro* and were therefore included in the treatment regime (see section 3.1). 8-Bromo cAMP is a cAMP analogue and was selected because it is readily absorbed by cells and partially resistant to phosphodiesterases. In addition, the combined effects of MPA plus PGE_2 or 8-Bromo cAMP were assessed. The experiment detailed in table 3.3 was designed to determine whether a continuous supply of treatments was required to sustain decidualisation in ESCs *in vitro*. Relative levels of prolactin mRNA will be analysed to assess this (see 3.2.5).

Table 3.2

Experiment 1: Decidualisation of ESCs (n = 4)		
Treatment	**Concentration**	**Incubation time**
Control	N/A	4, 8 and 12 days
MPA	10^{-6} M	
PGE_2	10^{-6} M	
PGE_2 + MPA	10^{-6} M + 10^{-6} M	
8-Bromo cAMP	250μM	
8-Bromo cAMP + MPA	250μM + 10^{-6} M	

Table 3.3

Experiment 2: Decidualisation of ESCs and treatment removal (n = 5)		
Treatment	**Concentration**	**Incubation time**
Control	N/A	5 days, 5 days followed by 5 days with treatment withdrawal, 10 days continuous treatment
PGE_2 + MPA	10^{-6} M + 10^{-6} M	
8-Bromo cAMP + MPA	250μM + 10^{-6} M	

3.2.3 Prolactin ELISA

Media collected from experiment 1 at the termination of the time-points, (4, 8 and 12 days), was assayed on a commercial Wallac DELFIA® Prolactin kit (PerkinElmer™ Life Sciences, Wallac Oy, Turku, Finalnd). The kit instructions were adhered to with the exception that 150μl of sample media was added in place of the suggested 25μl to increase the sensitivity of the assay.

3.2.4 IGFBP-1 ELISA

Media collected from experiment 1 at the termination of the time-points, (4, 8 and 12 days), was assayed for IGFBP-1 using a two-site sandwich ELISA (section 2.6.1) (R & D Systems). Plates were coated with the IGFBP-1 capture antibody and incubated according to the IL-15 ELISA method. The antibody was added at 2µg/ml and 100µl per well. The plates were washed prior to use and a standard curve added in single wells to each plate consisting of eight standards starting with a top standard of 8ng/ml. 1:2 dilutions were performed until the bottom standard of 0.06ng/ml was obtained. Buffer plus Tween (B+T) was used to dilute samples and NSB wells were added as 100µl of B+T. A quality control was made up at 700pg/ml from the IGFBP-1 standard and added in quadruplicate wells to each plate. All media samples to be tested were added in quadruplicate wells, 100µl per well. Plates were sealed and incubated for 2 hours on a plate shaker at room temperature. The plates were washed in wash buffer four times each and tapped dry. The IGFBP-1 detection antibody was then added at 0.1µg/ml and 100µl per well and incubated at room temperature for 2 hours on a plate shaker. Plates were washed as before and tapped dry. Streptavidin peroxidase conjugate (Roche) was diluted in B+T to 0.125U/ml and 100µl pipetted into all wells. Plates were sealed and incubated for 20 minutes on a plate shaker at room temperature. The plates were then washed as before and 100µl of substrate added per well. Plates were allowed to develop for 15 minutes before stopping the reaction with 50µl of 2N sulphuric acid per well. All plates were read on a plate reader at 450nM. The computer program Assay Zap was used to analyse the results and construct a standard curve against which the samples could be judged.

3.2.5 RNA Extraction and Q RT-PCR

The RNA was extracted and cDNA prepared from the ESC experiments 1 and 2. Prolactin, IGFBP-1, IL-15 and progesterone receptor mRNA levels were measured by Q RT-PCR as described in section 2.5.

3.2.6 Statistical Analysis

Q RT-PCR and ELISA data were analysed by the methods described previously in section 2.8.

3.3 Results

3.3.1 Decidualisation of ESC Studies

3.3.1a Prolactin mRNA Expression and Release by ESCs

The mRNA expression of prolactin after 4 days is almost 4-fold greater than controls with MPA or with 8-Bromo cAMP treatment. Treatment with a combination of MPA and 8-Bromo cAMP stimulated a 22-fold increase in prolactin mRNA levels ($P < 0.017$). Prolactin protein released into the media after 4 days was below the minimum detection limit (250pg/ml) of the ELISA. After 8 days of treatment the combined treatment of PGE_2 and MPA had caused a 40-fold rise in prolactin mRNA relative to controls. A 163-fold increase in prolactin mRNA was produced by treatment with 8-Bromo-cAMP and MPA in combination ($P < 0.006$). This was comparable with the ELISA data that recorded 3-fold and 9-fold increases in prolactin release with PGE_2 plus MPA and 8-Bromo-cAMP plus MPA ($P < 0.009$) treatment respectively. The same pattern of prolactin mRNA expression and protein release were observed after 12 days of treatment. PGE_2 plus MPA stimulated an 11-fold rise in mRNA and a 9-fold increase in protein levels. 8-Bromo-cAMP plus MPA triggered a 29-fold increase in prolactin mRNA levels ($P < 0.03$) and a 22-fold increase in protein release ($P < 0.028$). Modest rises in prolactin mRNA expression and release were produced by treatment with 8-Bromo cAMP alone.

Figure 3.3

Relative levels of prolactin mRNA expression after 4 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **a** $p < 0.017$.

Figure 3.4

Relative levels of prolactin mRNA expression after 8 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **b** $p < 0.006$.

Figure 3.5

Relative levels of prolactin mRNA expression 12 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **c** $p < 0.03$.

Figure 3.3

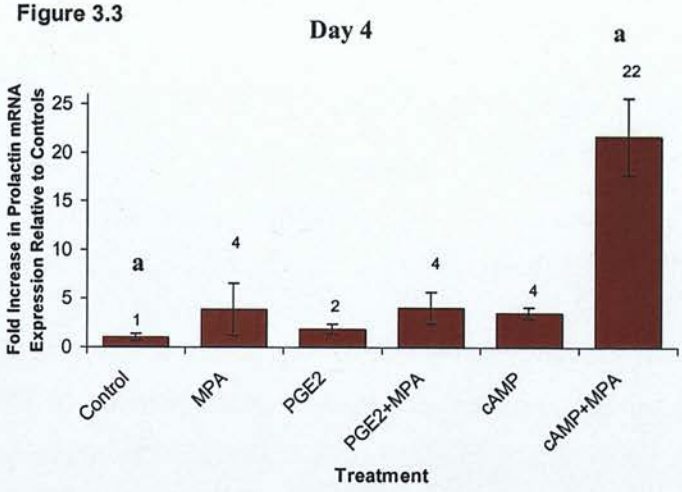


Figure 3.4

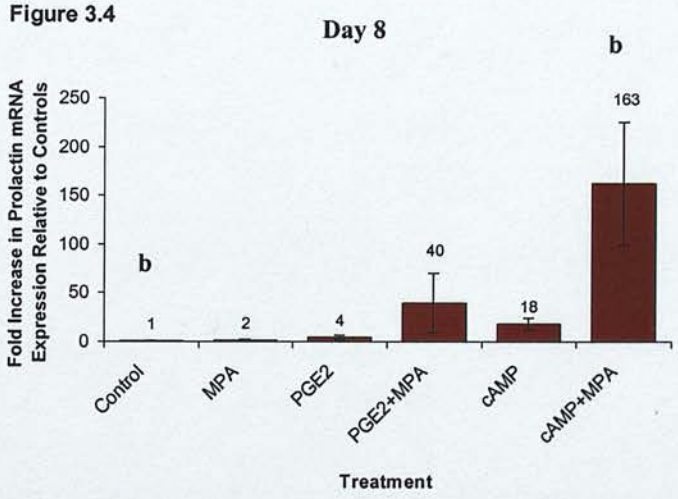


Figure 3.5

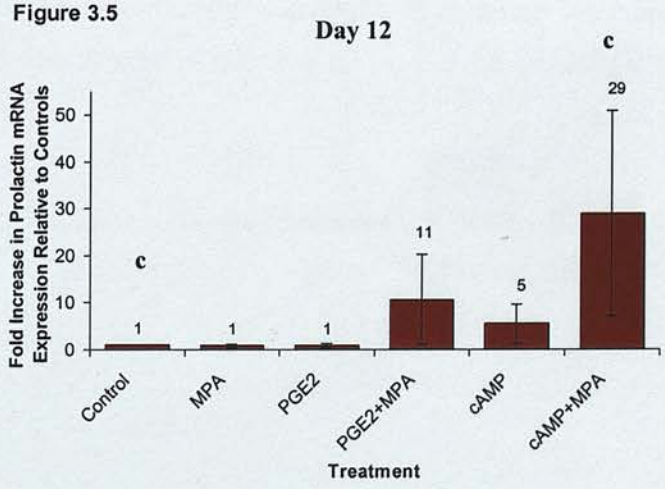


Figure 3.6

Prolactin protein release by human ESCs over 48 hours after 8 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. Same letters denote significant difference. **a** $p < 0.009$. The lower detection limit of the ELISA was 0.25ng/ml.

Figure 3.7

Prolactin protein release by human ESCs over 48 hours after 12 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. Same letters denote significant difference. **b** $p < 0.028$. The lower detection limit of the ELISA was 0.25ng/ml.

Figure 3.6

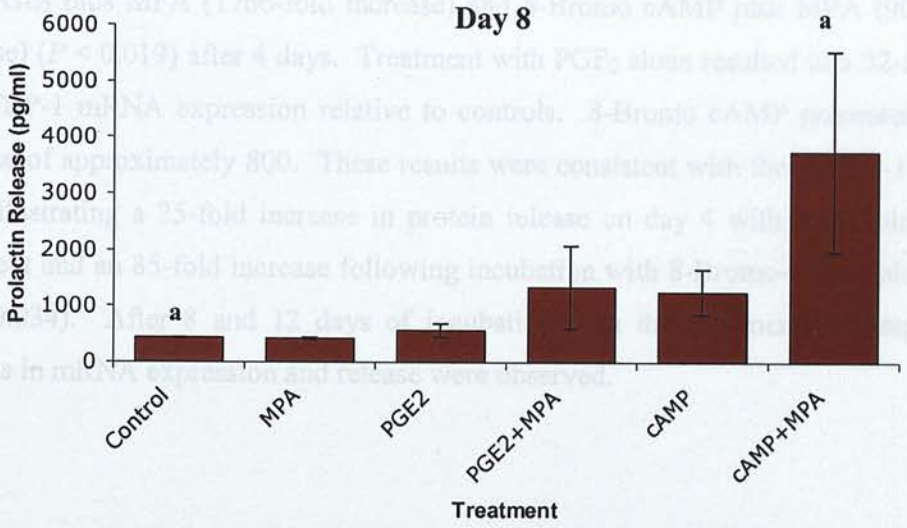
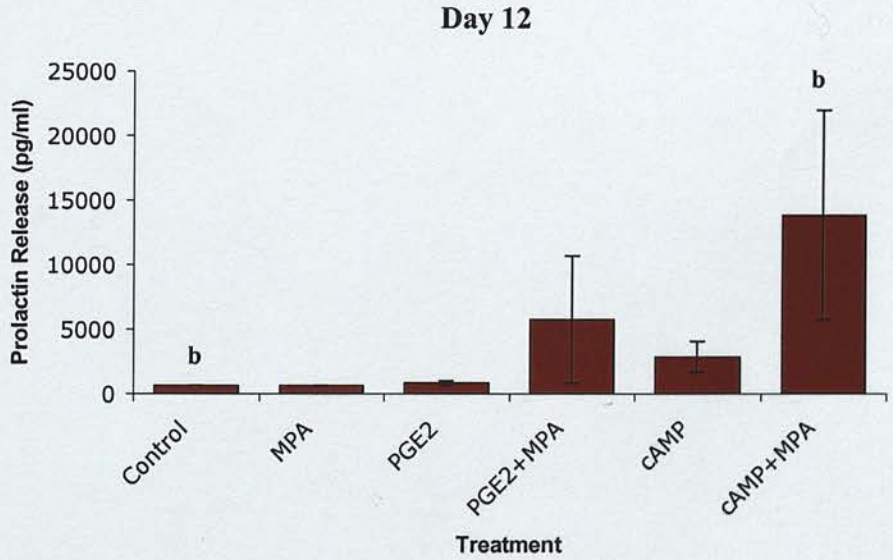


Figure 3.7



3.3.1b IGFBP-1 mRNA Expression and Release by ESCs

The mRNA expression for IGFBP-1 was increased considerably by treatment of ESCs with PGE_2 plus MPA (1766-fold increase) and 8-Bromo cAMP plus MPA (9075-fold increase) ($P < 0.019$) after 4 days. Treatment with PGE_2 alone resulted in a 32-fold rise in IGFBP-1 mRNA expression relative to controls. 8-Bromo cAMP promoted a fold increase of approximately 800. These results were consistent with the IGFBP-1 ELISA data, illustrating a 25-fold increase in protein release on day 4 with PGE_2 plus MPA treatment and an 85-fold increase following incubation with 8-Bromo-cAMP plus MPA ($P < 0.034$). After 8 and 12 days of incubation with the treatments corresponding patterns in mRNA expression and release were observed.

Figure 3.8

Relative levels of IGFBP-1 mRNA expression after 4 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **a** $p < 0.019$

Figure 3.9

Relative levels of IGFBP-1 mRNA expression after 8 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **b** $p < 0.015$,

Figure 3.10

Relative levels of IGFBP-1 mRNA expression after 12 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **c** $p < 0.017$.

Figure 3.8

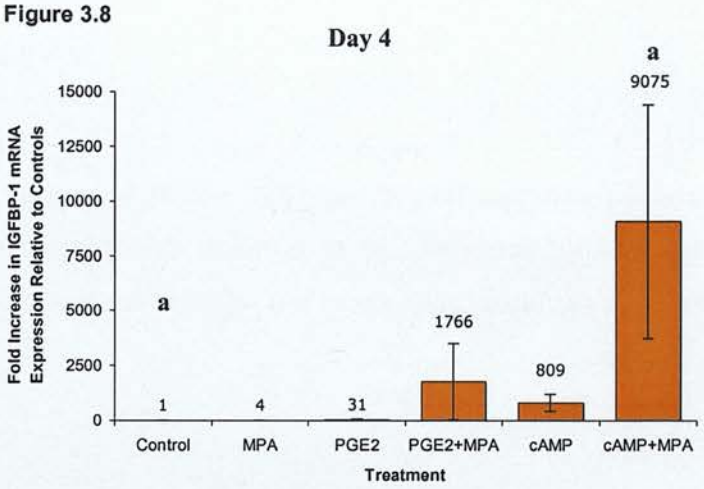


Figure 3.9

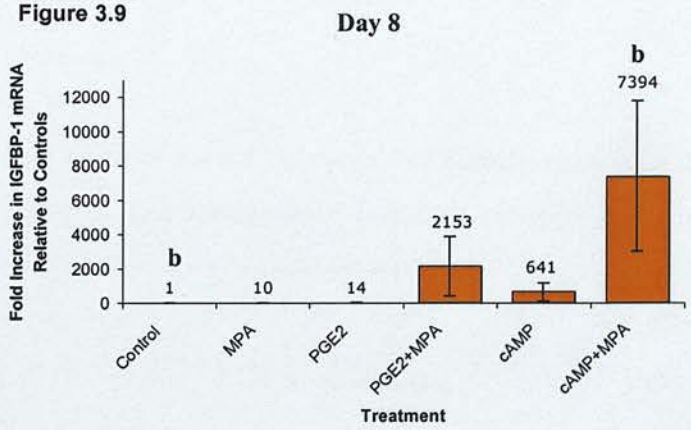
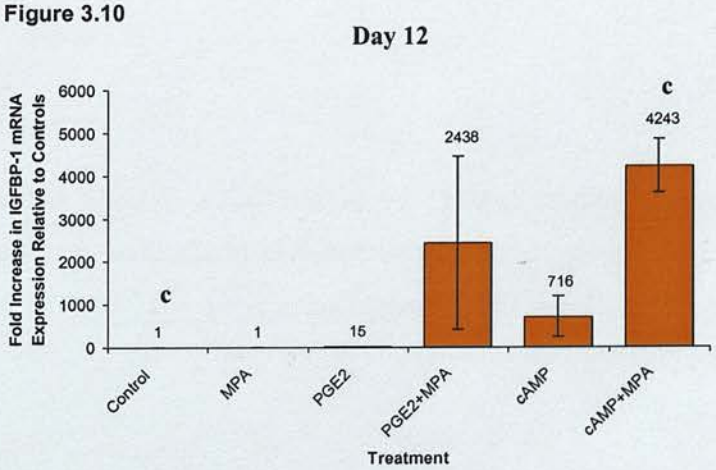


Figure 3.10



Figures 3.11

IGFBP-1 protein release (pg/ml) by human ESCs over 48 hours after 4 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. Same letters denote significant difference. **a** $p < 0.034$. The lower detection limit of the ELISA was 60pg/ml.

Figure 3.12

IGFBP-1 protein release (pg/ml) by human ESCs over 48 hours after 8 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. Same letters denote significant difference. **b** $p < 0.011$. The lower detection limit of the ELISA was 60pg/ml.

Figure 3.13

IGFBP-1 protein release (pg/ml) by human ESCs over 48 hours after 12 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. Same letters denote significant difference. **c** $p < 0.034$. The lower detection limit of the ELISA was 60pg/ml.

Figure 3.11

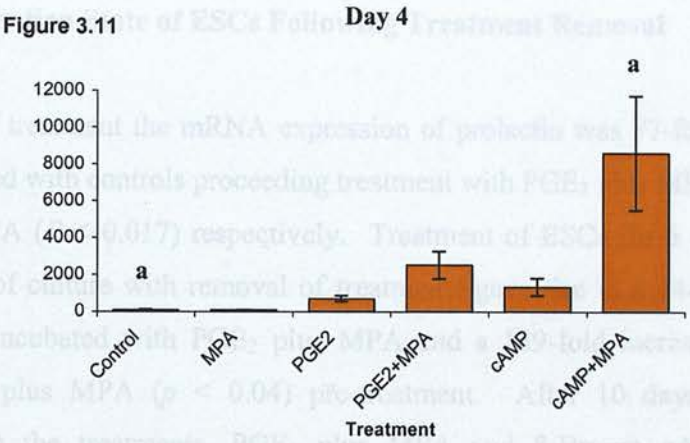


Figure 3.12

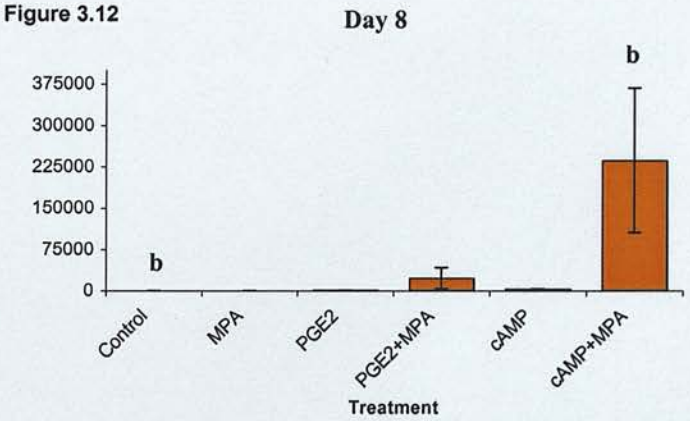
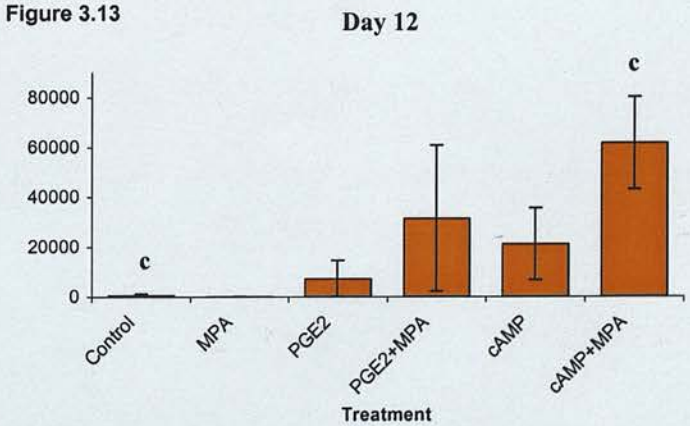


Figure 3.13



3.3.2 Decidualisation State of ESCs Following Treatment Removal

After 5 days of treatment the mRNA expression of prolactin was 77-fold and 261-fold greater compared with controls proceeding treatment with PGE_2 plus MPA and 8-Bromo cAMP plus MPA ($P < 0.017$) respectively. Treatment of ESCs for 5 days and then a further 5 days of culture with removal of treatments gave rise to a 44-fold increase in those initially incubated with PGE_2 plus MPA and a 139-fold increase following 8-Bromo cAMP plus MPA ($p < 0.04$) pre-treatment. After 10 days of continuous incubation with the treatments, PGE_2 plus MPA and 8-Bromo cAMP plus MPA stimulated 566- and 1187-fold ($p < 0.019$) rises in prolactin mRNA levels relative to controls.

Figure 3.14

Relative levels of prolactin (prl) mRNA expressed in ESCs treated for 5 days with decidualising stimuli. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **a** $p < 0.017$.

Figure 3.15

Relative levels of prolactin (prl) mRNA expressed in ESCs treated for 5 days with decidualising stimuli followed by 5 days of treatment withdrawal. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **b** $p < 0.04$.

Figure 3.16

Relative levels of prolactin (prl) mRNA expressed in ESCs treated for 10 days with decidualising stimuli. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **c** $p < 0.019$.

Figure 3.14

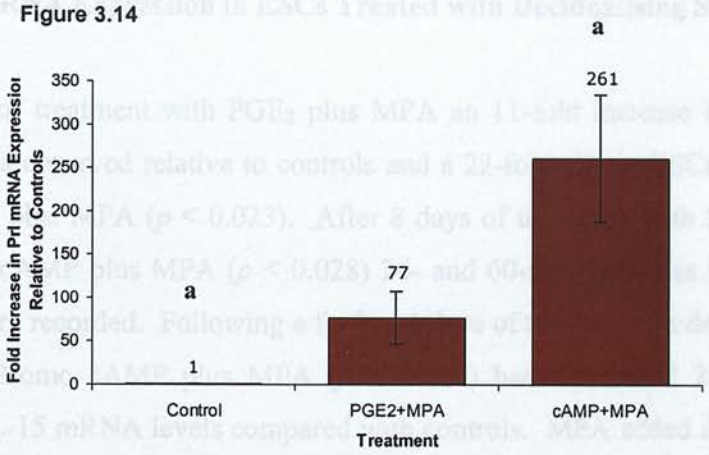


Figure 3.15

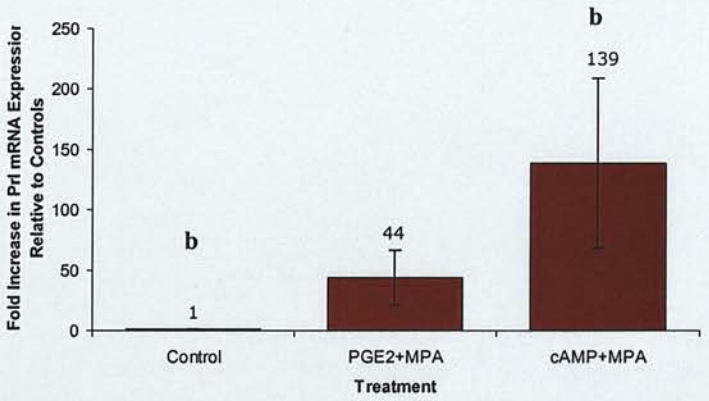
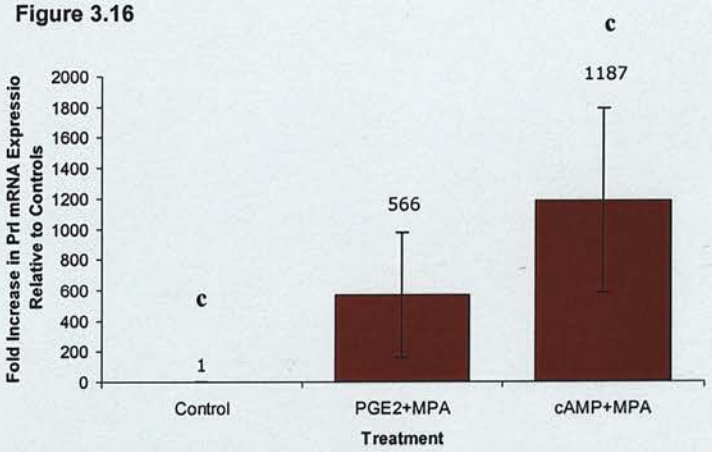


Figure 3.16



3.3.3 IL-15 mRNA Expression in ESCs Treated with Decidualising Stimuli *in vitro*

After 4 days of treatment with PGE_2 plus MPA an 11-fold increase in IL-15 mRNA expression was observed relative to controls and a 22-fold rise in ESCs treated with 8-Bromo cAMP plus MPA ($p < 0.023$). After 8 days of treatment with PGE_2 plus MPA and 8-Bromo cAMP plus MPA ($p < 0.028$) 26- and 60-fold increases in IL-15 mRNA expression were recorded. Following a further 4 days of treatment on day 12, PGE_2 plus MPA and 8-Bromo cAMP plus MPA ($p < 0.028$) had stimulated 20- and 168-fold increases in IL-15 mRNA levels compared with controls. MPA added alone produced a 2-fold increase in expression and 8-Bromo cAMP on its own stimulated 4-, 18- and 25-fold rises in IL-15 mRNA levels on days 4, 8 and 12 respectively.

Figure 3.17

Relative levels of IL-15 mRNA expression after 4 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **a** $p < 0.023$

Figure 3.18

Relative levels of IL-15 mRNA expression after 8 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **b** $p < 0.028$

Figure 3.19

Relative levels of IL-15 mRNA expression after 12 days of treatment with a variety of treatments designed to stimulate decidualisation in ESCs *in vitro*. The numbers located above the error bars indicate the fold increase in expression relative to controls. Same letters denote significant difference. **c** $p < 0.028$.

Figure 3.17

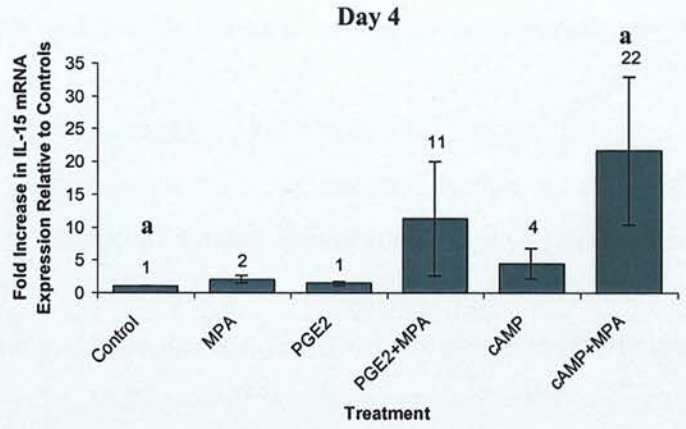


Figure 3.18

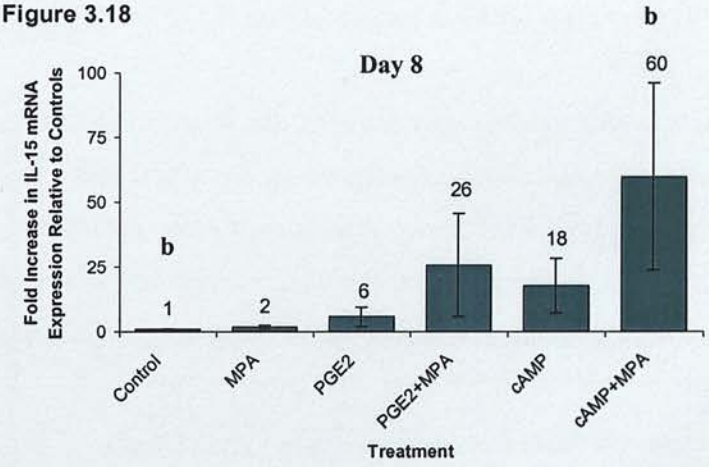


Figure 3.19

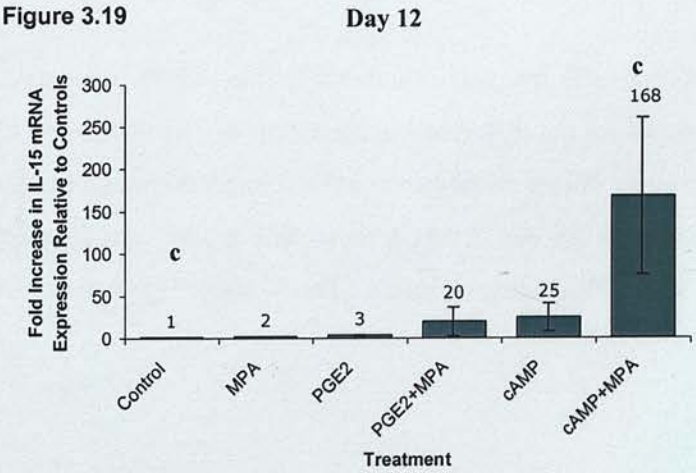


Figure 3.20 Correlation between prolactin and IL-15 mRNA levels on day 4

The correlation coefficient was calculated for the relative expression levels of IL-15 and prolactin mRNA for the different treatments on day 4 and found to be 0.92. This graph represents the log values of prolactin mRNA expression against the log values of IL-15 mRNA expression for the different treatment groups and illustrates the rise in IL-15 expression along with prolactin levels. The R^2 value is stated on the graph.

Figure 3.21 Correlation between prolactin and IL-15 mRNA levels on day 8

The correlation coefficient was calculated for the relative expression levels of IL-15 and prolactin mRNA for the different treatments on day 8 and found to be 0.97. This graph represents the log values of prolactin mRNA expression against the log values of IL-15 mRNA expression for the different treatment groups and illustrates the rise in IL-15 expression along with prolactin levels. The R^2 value is stated on the graph.

Figure 3.22 Correlation between prolactin and IL-15 mRNA levels on day 12

The correlation coefficient was calculated for the relative expression levels of IL-15 and prolactin mRNA for the different treatments on day 12 and found to be 0.97. This graph represents the log values of prolactin mRNA expression against the log values of IL-15 mRNA expression for the different treatment groups and illustrates the rise in IL-15 expression along with prolactin levels. The R^2 value is stated on the graph.

Figure 3.20 **Day 4**

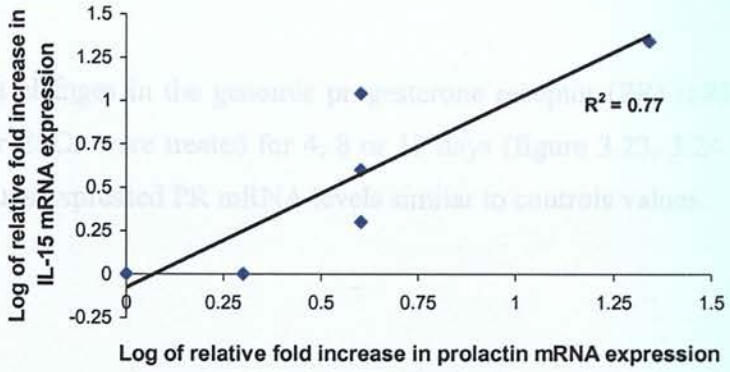


Figure 3.21 **Day 8**

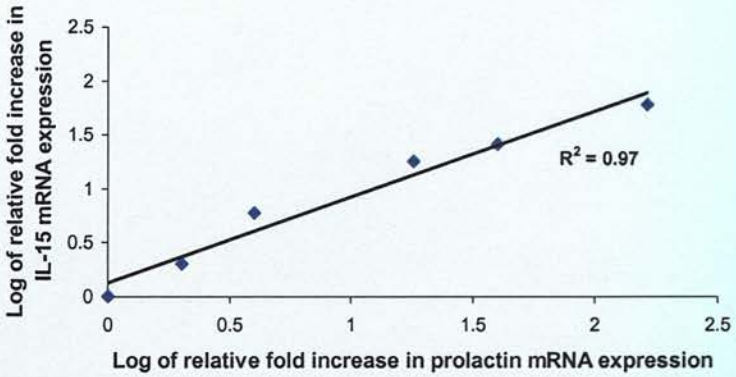
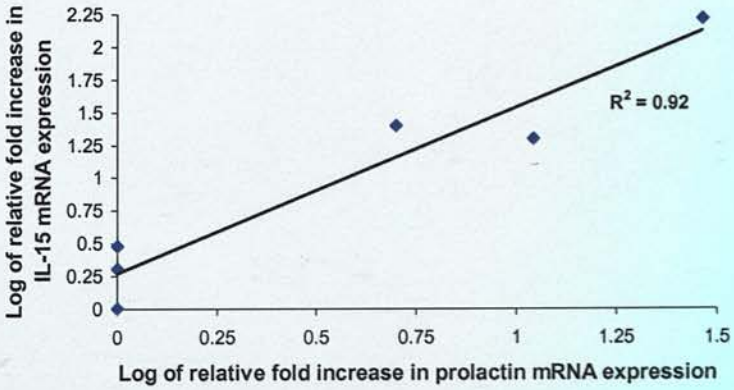


Figure 3.22 **Day 12**



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Day 4

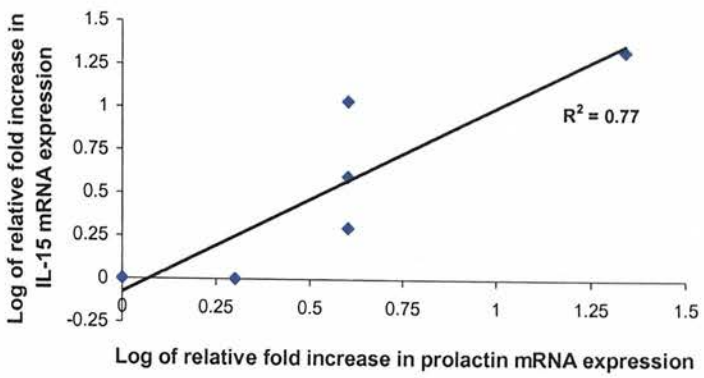


Figure 3.21

Day 8

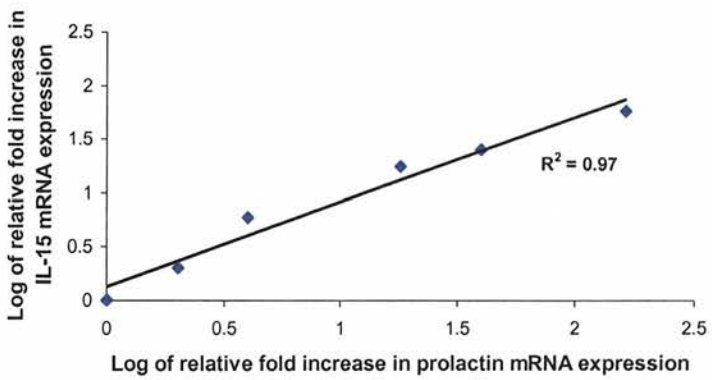
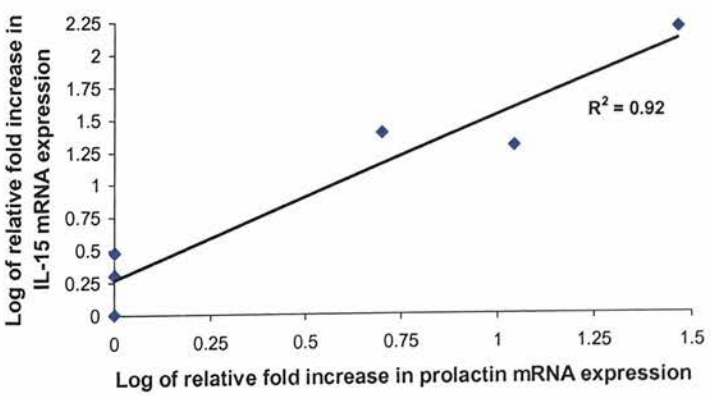


Figure 3.22

Day 12



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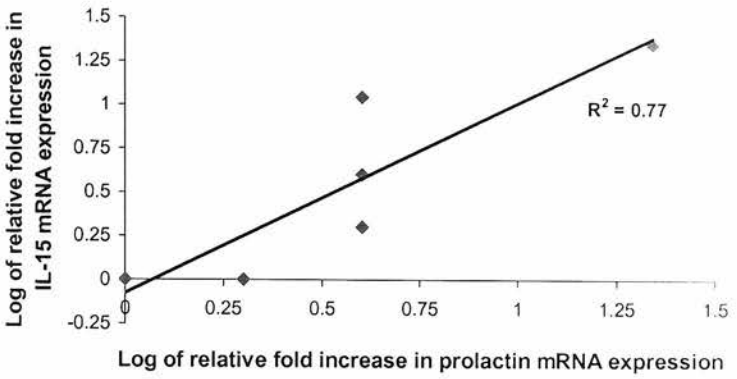


Figure 3.21

Day 8

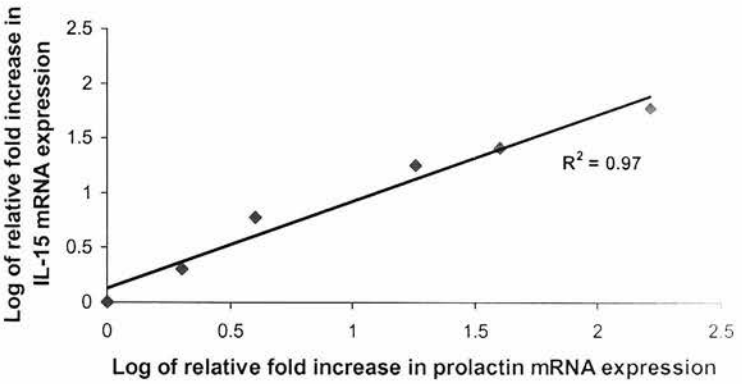
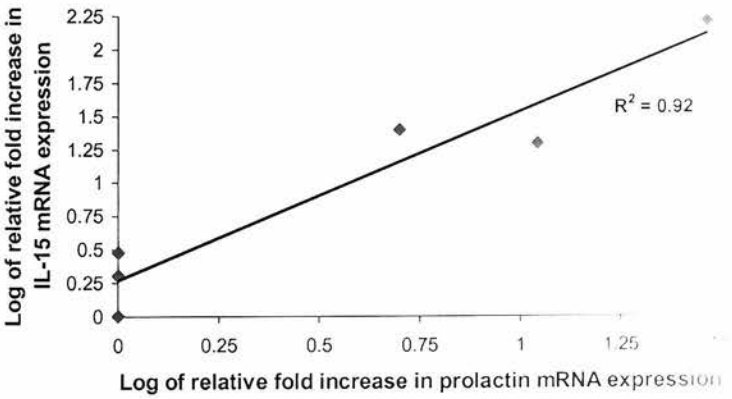


Figure 3.22

Day 12



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Day 4

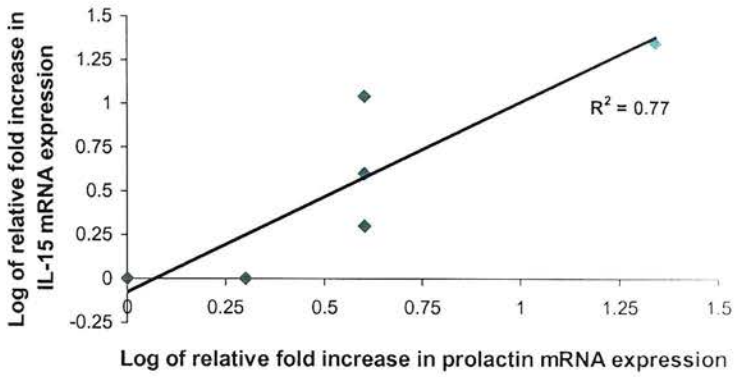

Figure 3.21

Day 8

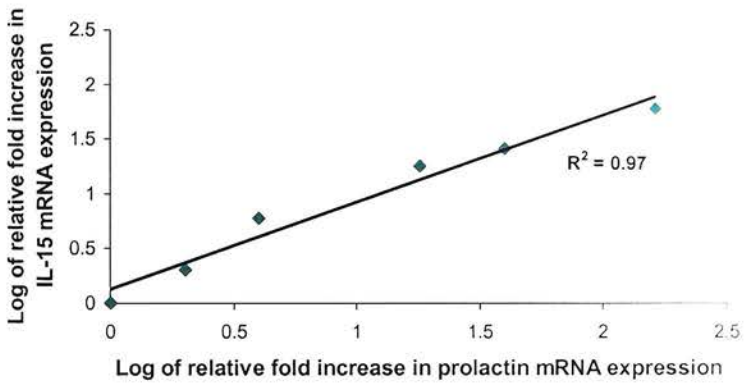

Figure 3.22

Day 12